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► To cite this version:

Soizik Berlivet, Jérémy Scutenaire, Jean-Marc Deragon, Cécile Bousquet-Antonelli. Readers of the m6A epitranscriptomic code. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 2019, 1862 (3), pp.329-342. 10.1016/j.bbagr.2018.12.008 . hal-02119233

HAL Id: hal-02119233

<https://univ-perp.hal.science/hal-02119233>

Submitted on 19 Dec 2019

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Readers of the m⁶A epitranscriptomic code

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ABSTRACT

N⁶-methyl adenosine (m⁶A) is the most prevalent, evolutionarily conserved, modification of polymerase II transcribed RNAs. By post-transcriptionally controlling patterns of gene expression, m⁶A deposition is crucial for organism reproduction, development and likely stress responses. M⁶A mostly mediates its effect by recruiting reader proteins that either directly accommodate the modified residue in an hydrophobic pocket formed by their YTH domain, or otherwise have their affinity positively influenced by the presence of m⁶A. We describe here the evolutionary history, and review known molecular and physiological roles of eukaryote YTH readers. In a second part we present non YTH-proteins, which roles as m⁶A readers largely remain to be explored. The diversity and multiplicity of m⁶A readers carried by most organisms together with the possibility to regulate their expression and function in response to various cues, offers a multitude of possible combinations to rapidly and finely tune cell gene expression pattern and hence plasticity.

1- What is an m⁶A Reader?

All types of RNAs carry post-transcriptionally added modifications that impact their processing, stability and function. Messenger RNAs (mRNAs) are no exception and can carry several types of chemical modifications, the most evolutionarily conserved and abundant of which consists in the addition of a methyl group at position N⁶ of internal adenosines, m⁶A for short. M⁶As are present in nearly all types of RNAs and can be found in most organisms from bacteria to animals [1].

M⁶As are co-transcriptionally deposited at the DRACH (D=A/G/U, R=A/G, H=A/C/U) consensus site on RNA polymerase II (pol II) transcribed RNAs (mRNAs, long non coding RNAs (lncRNAs), precursors to microRNAs (pri-miRNAs) and circular RNAs (circRNAs)) by an evolutionarily conserved methylase complex, known as the "writer" complex and can be reverted to unmodified As by demethylases of the ALKBH (Alkylation repair Homolog) family called "erasers" [2].

The biological consequences of m⁶A-mediated regulations are multiple but a common feature is that they are required for fundamental processes in every eukaryote. They are crucial for gametogenesis [3, 4], sex determination [5, 6], embryogenesis [7-9], cell fate determination [7, 10, 11], neuronal functions [6] and to control the cell circadian clock [12]. The m⁶A marks are also necessary for cell acclimation and recovery from stress [13-15]. They mediate their physiological roles by controlling mRNA fate (maturation, splicing, nuclear export, translation, stability and storage), pri-miRNA maturation [16] and/or lncRNAs functions in the cell [17]. At the molecular level, the most prevalent role of m⁶As is to influence the binding of proteins to their RNA targets (Figure 1). They can act to repel RNA Binding Proteins (RBPs) [18, 19] (Figure 1A) or as a signal to recruit RBPs called "m⁶A-readers" that convey the m⁶A signal (Figure 1). Once bound to their RNA targets, m⁶A readers can directly control the fate of their target transcripts and/or can do so by directly or indirectly recruiting effector proteins (Figure 1A).

What is an "m⁶A-reader"? The question is seemingly straightforward to answer when it comes to proteins that carry the conserved YTH-domain. Indeed, the YTH domain is a globular RNA binding motif that forms a hydrophobic pocket that directly accommodates and tightly binds the m⁶A residue. But recent findings provide proofs that the presence of m⁶A can also positively influence the recruitment of non-YTH RNA binding proteins to their targets, either by increasing their affinity for their methylated ssRNA binding region by an unknown mechanism or by exposing RBP binding surfaces

through alteration of RNA structures in a mechanism tagged as "m⁶A-switch" (Figure 1B, C) [17, 20]. M⁶A-related RNA structure alterations can expose single-stranded binding sites allowing readers binding (Figure 1B) [21, 22] or, if the modified A is situated immediately 5' or 3' to the RNA structure, stabilize the duplex (Figure 1C) [17]. In this latter case, proteins which binding is influenced by these m⁶A-stabilized structures, if any, are yet to be identified.

We will expose in a first chapter the current state-of-the-art on YTH-domain m⁶A readers molecular and physiological roles from animals, budding yeast and *Arabidopsis thaliana*. In a second part, we will give an overview on other types of m⁶A-readers which binding is positively influenced by the presence of m⁶A and that hence convey the m⁶A signal.

2- YTH-domain m⁶A Readers

Firstly identified on the human splicing factor YT521-B protein (now called YTHDC1) [23], the **YT521-B** homology domain (**YTH**) was subsequently recognized as highly conserved amongst eukaryotes and proposed to be a structured RNA binding domain [24, 25]. In vertebrates, proteins carrying a YTH domain were labeled as **YTH Domain Family** (YTHDF) or **YTH Domain Containing** (YTHDC) proteins, initially according to their subcellular distribution. Recently, Scutenaire *et al.* found that YTH domains fall into two evolutionary subclades respectively containing the vertebrate YTHDF1-3 proteins and the YTHDC1-2 proteins. Members of these subclades were hence tagged as DF and DC-type motifs [26].

2.1- Structural bases of m⁶A binding by the YTH domain.

Several studies reported the structure of YTH domains from yeast and animal proteins, of the DC and DF types, either alone or in a complex with an m⁶A-containing RNA aptamer [27-32]. A first conclusion that can be drawn from these studies is that all YTH domains adopt a conserved canonical fold consisting in three α helices and six β strands. The six β strands are organized in an atypical barrel fold that forms, with the packing of the three α helices against it, a hydrophobic core. DF and DC-type YTH domains use a conserved mechanism for m⁶A binding. In all cases, m⁶A is accommodated in a hydrophobic aromatic cage (formed by the C-termini of β 1, α 1 and β 2, the N-terminus of α 2 and the loop between β 4 and β 5) composed of three tryptophans (the WWW type

found in most YTHDFs), of two tryptophans and one tyrosine (the WWY type, found in several yeast YTHDFs) or of two tryptophans and one leucine (the WWL type, found in most YTHDCs). Although the nucleotides surrounding the m⁶A (in -2, -1, +1 and +2) contribute significantly to m⁶A binding intensity, no base-specific interaction (other than with the m⁶A) was observed in these studies. Also, the ways YTH hydrophobic cores accommodate nucleotides preceding and following the m⁶A are variable among studied proteins. A different selectivity at the -1 position was nevertheless observed for the human DC1 protein that favors G over A, which is not the case for other DC and DF-type YTH domains [30, 31]. Finally, in several situations, YTH domains were also shown to bind unmethylated RNAs, albeit with a much lower affinity [25, 27-29], leaving open the possibility that *in vivo*, YTH-domain proteins bind to non-m⁶A modified RNAs.

2.2- Evolutionary history of YTH-domain proteins in eukaryotes

The m⁶A writer complex can be found in all major eukaryotic lineages, suggesting that the m⁶A modification of RNA pol II transcripts is an ancestral mechanism that first evolved in the common ancestor of eukaryotes. YTH-domain containing proteins of both types (YTHDC and YTHDF) are also well represented in eukaryotes (Figure 2A), suggesting that these readers co-evolved simultaneously with m⁶A methylation capabilities. However, the evolution of YTH-proteins was apparently much more dynamic than m⁶A methylation capabilities, with many cases of extinctions and associations with other functional domains. For example, YTHDC and DF proteins can be easily found in Stramenopile, Haptophyte and Aveolate species but were apparently lost from the Excavate, Discicristate and Rhizaria lineages (Figure 2A). Since genomic data from these latter lineages are still scarce, it is possible that YTH readers are not completely absent from those, but they are certainly less represented compared to the three former lineages. Another example is the apparent absence of YTH-proteins in the Amoebozoa lineage, an outgroup of the Metazoans and Fungi where YTHDC and DF proteins are well represented. Finally, YTH-proteins are apparently absent from Rhodophytes, while only the DC version is present in Chlorophytes and both YTH types are present in Embryophytes. These examples suggest that one or both YTH versions were lost at different times and in different lineages during eukaryote evolution. Another interesting feature of eukaryote YTH evolutionary history is the tendency for this motif to fuse with other functional domains. This was the case in the Fungi lineage

where a DC domain from many Ascomycota and Basidiomycota species, fused with an RNA Recognition Motif (RRM) and a DF domain from several Pezizomycotina species fused with a protein kinase motif. Also, at two times independently, in Stramenopile and Embryophyte lineages, DC motifs fused with zinc fingers. In Stramenopiles, another conserved motif named DCD, is also present in combination with YTHDC and zinc finger domains. The DCD domain was first identified in plants where it was tentatively associated with development and programmed cell death [33]. Finally, in Metazoans, YTHDC motif can be found in fusion with helicase domains.

An interesting question is whether YTH domains kept their m⁶A binding capabilities in all eukaryotic lineages, especially following fusion with other functional domains. To answer this question, we aligned in Figure 2B, representative YTH sequences from all eukaryotic lineages and configurations represented in Figure 2A. Firstly, structure prediction algorithms anticipate a general conservation of the secondary structure elements for all YTH domains (Figure 2B). Secondly, they display a general conservation of the three amino acids forming the m⁶A-accommodating hydrophobic aromatic pocket (see above), although with some possible conservative changes. In three cases (Chlorophytes YTHDC and Alveolates YTHDF and DC), the third amino acid of the aromatic cage was replaced by a phenylalanine, which is likely to be a conservative change. In two other situations (Stramenopiles YTHDC and Fungi RRM-YTHDC), the middle tryptophan was replaced by a leucine. Since the substitution of one aromatic residue by a leucine was shown not to result in a significant affinity change for m⁶A [29], this combination is also likely to be functional for m⁶A binding. Finally, in the case of Alveolates YTHDC, the middle amino acid is an alanine. Mutating the central tryptophan for an alanine in the context of the human DC1, DF1, DF2 YTH domains and of the yeast *Z. Rouxii* DF (Mrb1) YTH was shown to severely disrupt their binding to m⁶A [27, 28, 30, 31]. Therefore, it remains to be seen if the divergent aromatic cage of the Alveolate YTHDC protein (of the WAF type) can still accommodate m⁶A. In conclusion, the alignment presented in figure 2B, suggests that the m⁶A binding capability was mainly conserved through eukaryote evolution. This, in turn, supports that the reading of the m⁶A epitranscriptomic code can be transduced differently in different lineages, depending on the nature of YTH-associated functional domains.

The presence of YTHDC and DF proteins in a given eukaryotic lineage does not imply that all species belonging to that lineage possess both types of readers. For example, in

Alveolates, all Ciliate species have only the DF-type while all other Alveolate species (including Apicomplexans and Dinoflagellates) only possess the DC-type. As an example of the dynamics of evolution of YTH proteins within a given lineage, we looked in more details at the situation in Viridiplantae (a monophyletic lineage including Chlorophytes and Embryophytes). Figure 3A presents the evolutionary history of the YTHDF type in Viridiplantae. A gene coding for a YTHDF protein was likely present in the common ancestor of Viridiplantae but was lost in the Chlorophyte lineage. In the common ancestor of angiosperms, a gene duplication event, likely followed by the neofunctionalization of the generated copies, gave rise to the FA, FB and FC subgroups, present in all modern angiosperm species. For YTHDC (Figure 3B), a single gene coding for YTHDC protein was likely present in the common ancestor of Viridiplantae as well as an independent gene coding for a zinc finger protein. For Chlorophytes, this *YTHDC* gene was lost in the *Micromonas* and *Ostreococcus* lineages but conserved in the *Chlamydomonas* and *Volvox* lineages. In the common ancestor of Embryophytes, two YTHDC versions (DC1 and DC2) evolved from this ancestral YTHDC sequence from a gene duplication event. In the DC1 version, the YTH domain was fused to the zinc finger domain that ceased to be coded independently from YTH in most plants. Subsequently, YTHDC2 was lost in the monocot lineage, while conserved in the eudicot lineage. The complex evolutionary history of YTH genes in Viridiplantae, that includes several gene loss, duplication, fusion and neofunctionalization events is representative of the highly dynamic nature of YTH evolution in eukaryotes and is probably reflecting the plastic ways to read and transduce the m⁶A epitranscriptomic signal resulting from distinct selection pressures in different lineages. It is intriguing to observe that species with clear m⁶A capability (like several Rhodophyte species or microalgae of the *Micromonas* and *Ostreococcus* genders, for example) do not code for any YTH reader, suggesting that, in these cases, other types of m⁶A readers may fulfill this function.

2.3- Molecular and physiological roles of YTH domain proteins as m⁶A readers

Mammalian genomes code for five YTH domain proteins, three belonging to the DF group and two to the DC group [34]. In *Drosophila melanogaster*, only two YTH-domain proteins exist: YT521-B, the homolog of YTHDC1, and CG6422, the sole member of the YTHDF family [35]. The *Saccharomyces cerevisiae* genome codes for a single YTH-reader

of the DF type, while plant genomes contain large families of YTH-proteins of both types; for example *Arabidopsis thaliana* carries thirteen putative such readers. So far, most studies on YTH domain m⁶A readers focused on mammalian proteins but YTH domain proteins also perform essential functions in fruit fly, zebrafish, *Arabidopsis* or *Saccharomyces cerevisiae* (table 1).

In animals, YTH domain m⁶A readers mediate many molecular aspects of RNA metabolism driven by m⁶A modifications including mRNA splicing, nuclear export, stability or translation control. Similarly, they participate in the m⁶A-controlled regulation of physiological processes and are involved in viral infection responses, diseases (including cancer) or sterility defects.

2.3.1- Molecular roles of animal YTH domain m⁶A readers

The most prevalent and general role of the m⁶A-mark is to promote translation and induce mRNA destabilization [36]. Nonetheless, on a subset of transcripts and/or according to the cellular or environmental context, m⁶A and its YTH-readers may regulate other post-transcriptional events such as alternative splicing or have an antagonist impact on translation or mRNA stability control (Figure 4).

Splicing

Before being characterized as an m⁶A binding protein, mammalian YTHDC1 and its YTH domain were identified as being required for some alternative splicing events [25] and this effect was latter found to be dependent upon YTHDC1 ability to accommodate m⁶As [37]. In *Drosophila*, YTHDC1 is essential for the correct splicing of sex-lethal (*Sxl*) and subsequently for the proper regulation of sex-specific genes. In male, alternative splicing of the *Sxl* pre-mRNA prevents the production of a full-length protein as it retains an exon containing a premature stop codon that triggers its decay. In female, m⁶A-modified residues recruit YTHDC1, which in turn promotes the exclusion of the male-specific exon and consequently the expression of the functional *Sxl* protein [5, 6, 35]. While depletion of YTHDC1 favors the production of the male-specific isoform, its overexpression leads to a male lethality phenotype, which can be rescued by deletion of *Ime4*, the fruit fly active methyltransferase of the writer complex. These data confirm the m⁶A-dependent function of YTHDC1 in sex determination in *Drosophila*.

In mammals, pre-mRNAs bound by YTHDC1 can competitively recruit two splicing effectors: the serine/arginine-rich splicing factors 3 and 10 (SRSF3 and SRSF10)[37]. SRSF3 and 10 are general splicing factors that act to promote exon inclusion and exon skipping, respectively. Nonetheless, consistently with the observation that the m⁶A-mark does not globally regulate splicing [36], only a subset of SRSF3 and 10 target mRNAs carry YTHDC1 binding sites or m⁶A modification [37]. This suggests that the m⁶A/m⁶A-reader axis solely acts on a small subset of alternative splicing events that can nonetheless reveals to be crucial as exemplified by the regulation of *Sxl* splicing in fruit fly.

In addition, SRSF3 seemingly transduces differently the m⁶A-driven YTHDC1 signal depending on its phosphorylated state. Indeed, on the one hand, hypophosphorylated SRSF3, which is the export-competent form of SRSF3, promotes the export of at least some of YTHDC1 m⁶A-targets [38]. SRSF3 is essential for this function as only transcripts bound by both YTHDC1 and SRSF3 accumulate in the nucleus following YTHDC1 knockdown while transcripts bound by YTHDC1 but not SRSF3 do not. On the other hand, the phosphorylated form of SRSF3 competes with SRSF10 to bind YTHDC1 and promote exon inclusion, while SRSF10 favors exon skipping. Therefore, SRSF3 may act as a specific YTHDC1 effector for splicing and nuclear export. These studies provide an example where a unique m⁶A reader induces different outcomes depending on its effector protein modifications.

mRNA stability

In mammals, YTHDF2 is one of the readers decoding the m⁶A signal that triggers mRNA destabilization. In HeLa cells, YTHDF2 decodes the m⁶A-mark on more than two thousand mRNAs and its deletion induces a significant increase of its target levels [39]. The stability of transcripts targeted by the m⁶A/YTHDF2 axis is negatively regulated at the step of deadenylation. Indeed, mRNAs bound by YTHDF2 tend to possess shorter poly(A) tails compared to the pool of non-YTHDF2 targets [39] and YTHDF2, through its N-terminal domain, promotes deadenylation when tethered to a reporter transcript [40]. This effect is most likely mediated by the m⁶A mark as the knockdown of the METTL3 methylase reduces deadenylation in an *in vitro* reporter assay. The effector of YTHDF2-mediated deadenylation is the CCR4/CAF/NOT complex recruited to mRNAs via a direct interaction between YTHDF2 N-terminus and the superfamily homolog (SH)

domain of CNOT1, the scaffolding component of the deadenylation complex [40]. YTHDF2 was initially proposed to trigger mRNA decay by promoting their exit from polysomes and targeting to p-bodies (cytosolic mRNP aggregates containing also mRNA decay factors) [39] which until recently were proposed as mRNA decay centers. Nonetheless, recent advances invalidated this model by showing that no significant decay occurs in p-bodies that might rather serve as storage and triage center of mRNPs [41]. This leaves open the question of the significance of YTHDF2-induced mRNA aggregation into p-bodies.

The role of YTHDF2 in mRNA stability regulation control seems to be partially conserved as similar destabilization effects were observed in *Saccharomyces cerevisiae*, mouse and zebrafish [8, 42]. In zebrafish, Ythdf2 facilitates the degradation of maternal transcripts during the maternal-to-zygotic transition [8]. Similarly, YTHDF2 is maternally required in mouse for appropriate transcript dosage [43]. However, while in zebrafish the degradation of maternally provided transcripts occurs early in embryo development, it starts in mouse during oocyte maturation. YTHDF2 is therefore necessary for both oocyte competence and early zygotic development.

Finally, YTHDF2 may also take part in the regulation of atypical RNAs. Indeed, in human cells, it interacts with more than one thousand circRNAs. Circular RNAs are the product of back-splicing events that are promoted by the formation of a loop resulting from the base pairing between intronic repeats. Circular RNAs can act as "decoys" to neutralize miRNAs, or to sequester RNA binding proteins. Recently they were found to be translated into proteins and to carry m⁶A modifications that influence their production and translation [44, 45]. In cells depleted of YTHDF2, the precursors to circRNAs have half-lives significantly shorter than that of any other type of m⁶A-carrying transcripts. In addition, the m⁶A marks do not influence the stability of mature circRNAs. Although a link between YTHDF2 role on precursors to circRNAs and its m⁶A-reading activity, yet remain to be demonstrated, these observations point to a function of this protein into the maturation and/or stability control process of precursors to circRNAs [44, 45].

Other m⁶A readers are also involved in the regulation of mRNA stability, dependently or independently of YTHDF2. For example, YTHDF1 and DF3 can help to target methylated transcripts to YTHDF2 for degradation in HeLa cells [46]. Moreover, an increase of the m⁶A/A ratio in mRNA was observed after 24h overexpression of YTHDF1 highlighting a potential role of YTHDF1, this time in mRNA stabilization [47].

Translation

In mammals, the regulatory role of m⁶A on translation can be decoded by several YTH-readers, which act on specific transcripts and/or in certain cell types or environmental conditions.

YTHDF1 is seemingly mediating a global promoting effect of m⁶A on translation, at least in certain cell types or organisms [47, 48]. In mouse it is required, together with the m⁶A mark for global protein synthesis after peripheral sciatic nerve lesion [48]. In HeLa cells, this reader binds in an m⁶A-dependent manner to several thousand mRNAs and acts to recruit the translation initiation complex eIF3 (eukaryotic initiation Factor 3), which in turn binds mRNA in 48S complexes, and acts to organize translation initiation factors and ribosomes for productive translation [47]. In HeLa cells, at least YTHDF1 and 3 cooperate to stimulate the translation of 60% of YTHDF1 targets [46]. *In cellulo*, YTHDF3 facilitates the translation of its mRNA targets but neither directly nor alone [46]. Indeed, tethering assays show that its direct recruitment has no effect on the expression of a reporter transcript but that it stimulates YTHDF1-promoted translation [49]. In addition, its depletion induces a decreased binding specificity of YTHDF1 and YTHDF2 to their targets [49]. Therefore, it was proposed that YTHDF3 contributes to the RNA binding specificity of YTHDF1, hence indirectly promoting translation in an m⁶A-dependent manner. In addition, considering that YTHDF2 binding to its m⁶A targets is dependent upon YTHDF3, this reader is also likely to indirectly promote mRNA decay. Since in HeLa cells, YTHDF1 and 2 have 50% of their mRNA targets in common, they are likely to have antagonistic roles hereby regulating the balance between translation and degradation [46].

The translation mechanism of circRNAs is fundamentally distinct from that of linear mRNAs, as it requires the non-canonical eIF4G2 protein similarly to the translation initiation process of IRES-containing mRNAs [44]. The translation of circRNAs is also promoted by m⁶A but conversely to other transcripts, their mark is not decoded by YTHDF1 but by YTHDF3, which acts to recruit eIF4G2 [44].

Several lines of evidence suggest that the m⁶A reader YTHDC2 could participate in translation regulation. In mouse testis, its inactivation leads to decreased protein level of at least two of its targets [50]. Moreover, the tethering of YTHDC2 in an *in vitro* reporter assay pinpoints to the ability of this reader to promote translation. This role seems to depend on m⁶A modifications as the knockdown of METTL3 decreases the translation

efficiency of YTHDC2 targets. Furthermore, the depletion of YTHDC2 in human colon adenocarcinoma cells leads to the reduced translation of 300 genes, a phenotype that yet remains to be directly linked to the loss of YTHDC2-m⁶A-reading capability [51].

In mammalian cells, the m⁶A-mark deposited on 5'-UTRs of heat-induced transcripts, promotes their non-canonical, cap-independent, translation [14, 15]. YTHDF2, is proposed to participate in this process by protecting in the nucleus, the m⁶A residues deposited on these stress-response mRNAs until they are exported to the cytoplasm where these marks are decoded by a non-YTH reader [14, 15] (see chapter 4 on "other m⁶A readers").

2.3.2- Cellular and physiological roles of YTH-domain m⁶A readers in animals

Neuronal functions

YTH domain m⁶A readers are involved in neuronal functions at least in *Drosophila* and mouse. In *Drosophila*, YTHDC1 is highly expressed in the nervous system consistently with the high m⁶A levels. Flies depleted of YTHDC1 or Ime4 show similar behavior defects in flying and climbing and exhibit held-out wings [5, 6, 35]. These results indicate that these neuronal flaws are probably due to the loss of m⁶A modifications and YTHDC1 m⁶A-reading ability. In mouse, YTHDF1 is required to regenerate axons after peripheral sciatic nerve lesion, a phenotype here again also observed in hypomethylated mutants supporting the existence of an m⁶A/YTHDF1 axis important for the injury response of the peripheral nervous system [48].

Sex determination

The m⁶A binding YTHDC1 protein facilitates sex determination and dosage compensation in several organisms. In *Drosophila*, consistently with its role in the alternative splicing event that drives the expression of Sxl in female but not in male, the inactivation of Ythdc1 results in reduced female viability as it is the case in flies depleted for Ime4, the catalytic subunit of the m⁶A writer complex [5, 6, 35]. This phenotype is not fully penetrant as female viability decreases by 30%, consistently with the observation that loss of Ythdc1 or of the m⁶A mark results in a 50% decrease of the female specific isoform of Sxl [5, 6]. Removal of maternal Ythdc1, together with zygotic heterozygosity for Sxl, drops female viability to some 10% and results in sexual

transformations with appearance of male characteristics in females. In mammalian cell systems, the lncRNA XIST, which carries at least 78 m⁶A residues, is required for the transcriptional silencing of one of the X chromosomes and hence provides dosage equivalence between male and female during female development [52]. YTHDC1 shows a clear m⁶A-dependent binding to XIST and is sufficient to promote the XIST-mediated silencing of X-chromosome genes. Based on this result, one expects that knocking down YTHDC1 in mouse should have a strong impact on embryonic development, an experiment yet to be performed.

Stem cell state maintenance and differentiation

Regulation of stem cells state maintenance and differentiation is crucial for proper embryonic development. In zebrafish, the methyltransferase Mettl3 is required to inhibit the endothelial Notch signaling and to allow hematopoietic stem/progenitor cell differentiation [53]. Ythdf2 is the mediator of this effect: it recognizes the m⁶A-modified *notch1a* mRNA and leads to its degradation. In agreement with these results, Ythdf2 was found, in mouse, to bind pluripotent gene transcripts whose stability is increased in the absence of Mettl3 leading to an “hyperpluripotent” phenotype and to the disability of embryonic stem cells to properly differentiate [54].

Gametogenesis

Consistently with its high expression in mammalian germlines [55-57], YTHDC2 is required for proper male and female fertility. *Ythdc2*-deficient mice are viable but infertile with males and females having smaller testes and ovaries respectively. More specifically, males lack spermatozoa in the seminiferous tubules and epididymis while females lack follicles or have only early-stage follicles. In *ythdc2*-deficient mice, male and female germ cells fail to execute the meiotic prophase and are eventually removed by apoptosis [55-57]. These phenotypes are similar to that of mice depleted of MEIOC (Meiosis specific with Coiled-coil domain), a direct interactant of YTHDC2 [58, 59]. The gametogenesis defects of *ythdc2* and *meioc*-depleted mice is the consequence of defective meiosis programs which are characterized by an arrest at the meiotic prophase I and numerous abnormal metaphases [55, 57-59]. YTHDC2 and MEIOC are required to maintain the proper transcriptome needed for early meiotic progression. In both male and female germ cells, they act together to downregulate a set of mRNAs

coding for mitotic regulators; suggesting that these proteins might cooperate to antagonize mitosis [59]. In addition, YTHDC2 and MEIOC positively control the levels of mRNAs coding for meiosis-promoting proteins [57, 58], but in that case it is not clear if they act together to achieve this. Strikingly, in testis, transcripts that are most enriched in m⁶A marks are negatively regulated at the steady-state level by YTHDC2, suggesting that this reader could convey a destabilization signal on at least some of these mRNAs [57]. Consistently with this hypothesis, YTHDC2 is in a complex with the 5'-3' exoribonuclease XRN1 and with UPF1, a master regulator of the non-sense mediated decay [57] and was found *in cellulo* to destabilize a reporter transcript to which it was artificially tethered [50]. But YTHDC2 might also positively regulate translation. Indeed in the same tethering assay, it stimulates the translation of the reporter construct [50] and was found in testes, to interact with factors of the translation initiation machinery [57]. In *cellulo*, knockdown of the methyltransferase METTL3 decreases the translation efficiency of YTHDC2 target mRNAs indicating that, at least part of YTHDC2 effect on translation, is driven by its ability to bind m⁶A modifications [50]. Whether YTHDC2 stimulatory effect on translation is necessary for proper meiosis entry and gametogenesis yet remains to be determined.

YTHDC2 appears to convey two different molecular roles of the m⁶A signal: translation enhancement and mRNA degradation, putatively depending on the interactor/effector it recruits to its target transcript. This is an illustration of the complexity of the m⁶A “code” driven by multiple interactors/effectors of the YTH domain proteins.

Stress response

Several studies suggest that YTH domain m⁶A readers may significantly contribute to the cellular response to stress. As presented above, YTHDF2 indirectly promotes the cap-independent translation of heat-induced mRNAs, such as chaperones, suggesting that it is likely to be required for the acclimation and survival to environmental stresses [15]. The m⁶A-mediated cap-independent translation of stress-induced mRNAs is likely to exist in other stresses. Indeed, a global increase of mRNA-5'UTR m⁶A deposition has been described after UV exposure. However in that case, the role of these 5'-UTR m⁶As and the nature and function of their readers is yet to be investigated [60].

In heat-stress, the translation of circRNAs was found to increase in an m⁶A-dependent manner [44]. As YTHDF3 is involved in promoting the m⁶A-dependent translation of

circRNAs under normal growth conditions, it would be interesting to define whether it also plays a role in their heat-triggered expression.

2.3.3- Roles of YTH-domain m⁶A readers in animal diseases

With the growing evidences that YTH domain m⁶A readers play major role in diverse cellular and physiological mechanisms, their involvement in disease development starts to be investigated. In this part, we focus on the potential roles of YTH-domain proteins in sterility, viral infection and cancer.

Sterility

In Human, the level of m⁶A residues is negatively correlated with sperm mobility and positively correlated with sperm immotility [61]. An abnormal increase in RNA m⁶A levels was recently recognized as an augmented risk factor for asthenozoospermia, a disease which consequences are a reduced sperm motility that is a common cause of infertility. This increase of m⁶A content might be the consequence of the upregulation of METTL3 and METTL14 (a degenerated methyltransferase that acts as a cofactor of the writer complex) genes. How at the molecular and cellular level increase m⁶A content can trigger this sperm disease remains to be explored. But while there is no increase in YTHDF2 in asthenozoospermia patients, its mRNA expression correlates with the m⁶A content of sperm RNA, leaving open the possibility that this reader, or the lack of sufficient amount of this reader could be one of the causes of this type of sperm infertility.

Viral infection

Viruses are known to carry m⁶A-modified residues since the 70's but how this modification affects virus infection was unknown. Since then, m⁶A marks have been precisely mapped on different viruses such as HIV-1, the Influenza A Virus (IAV), the Zika Virus (ZIKV), the polyomavirus Simian Virus 40 (SV40) or the Hepatitis C Virus (HCV) [62-67]. Methyltransferase complex components are required for HCV and IAV to produce viral proteins while they inhibit the expression of ZIKV envelop protein. While, it is surprising that cytoplasmic viruses like HCV and ZIKV are regulated by m⁶A modifications as the writer complex is essentially found in the nucleus, components of the methyltransferase machinery can also be found in the cytoplasm of Huh7 and 293T

human cells [66]. Moreover, a functional link has been established between m⁶A readers of the YTHDF family and the regulation of infection from several viruses. Especially, YTHDF2 has been involved in viral infection control in several cases but the molecular role of this reader seems to diverge depending on the virus [65-67].

Contentious results have been obtained regarding the HIV-1 virus. It is clear that YTHDF1-3 bind the HIV m⁶A-modified RNA genome. However, Kennedy *et al.* found that YTHDF proteins overexpression enhances HIV gene expression, proteins and replication potential of the virus [62]. These data are different from the data obtained by Tirumuru *et al.* who found that YTHDF proteins overexpression inhibited HIV-1 infection and that their depletion inhibits HIV-1 gene expression [63]. These inconsistencies need to be resolved before the exact function of m⁶A readers in HIV-1 infection and replication and more generally in viral infections can be clearly defined.

Cancer

Deregulation of m⁶A RNA methylation and components of the m⁶A modification pathway have been associated with cancers (reviewed in [68]). So far, two YTH domain proteins have been associated with cancer: YTHDC2 and YTHDF2. In hepatocellular carcinoma cells (HCC), YTHDF2 mRNA level is high and its knockdown reduces HCC growth and metastasis. In fact, YTHDF2 recognizes the m⁶A modified *SOCS2* transcript to promote its degradation [69]. This regulation is essential for HCC proliferation as depletion of *SOCS2* partially restores the proliferation rate of METTL3-depleted HCC cells. In pancreatic cancer, YTHDF2 is upregulated and associated with patients' poor prognosis. Chen *et al.* established that while YTHDF2 promotes proliferation of pancreatic cancer cells, it has the opposite effect on cancer cell invasion and adhesion. Therefore, they proposed a dual role for YTHDF2 in pancreatic tumor progression: a positive role in proliferation and a negative role in invasion [70]. YTHDC2 mRNA level is high in several cancer cell lines including HCC. YTHDC2 knockdown inhibits human hepatocellular carcinoma proliferation and decreases the cell motility of colon tumor cell lines [51, 71]. Moreover, the downregulation of YTHDC2 reduces the number of liver metastasis when colon tumor cells are transplanted into the spleen of nude mice. In these cells, three hundred putative target genes of YTHDC2 were significantly increased in the 40S fraction suggesting that YTHDC2 may affect translation in cancer cells. Finally, a positive

correlation between cancer progression with metastasis and YTHDC2 expression was found.

The role of YTH m⁶A readers in cancer development and more generally of m⁶A pathway components clearly needs to be further investigated as these first studies suggest that they play critical roles in cancer development and progression.

2.3.4- The YTH domain m⁶A readers from *Arabidopsis thaliana*

As in most eukaryotes, m⁶A residues on RNA pol II transcripts were found to be present in *Arabidopsis* and to be deposited at the DRACH consensus by a writer complex containing the same subunits as the mammalian writer complex [9, 72-74]. The total loss of m⁶A arrests embryogenesis and is lethal at the adult stage [7, 72, 73]. Hypomethylated mutants display pleiotropic phenotypes including growth delay, aberrant shoot apical meristem proliferation, reduced root growth and aberrant gravitropic responses [7, 72, 73]. *Arabidopsis* contains several members of the ALKBH family, two of them (ALKBH9B and ALKBH10B) carry m⁶A demethylase activities on pol II transcripts and are respectively involved in the defense against viral infections [75] and in the control of floral transition and vegetative growth [74].

The YTH domain from *Arabidopsis* was initially identified on two proteins found to directly bind the CIPK1 (Calcineurin B-like-Interacting Protein Kinase 1) calcium-dependent kinase. Eleven *Arabidopsis* proteins were identified as sharing a highly conserved C-terminus, later tagged as the YTH domain, and were called ECT1 to 11 (for Evolutionarily Conserved Terminus) [76]. Subsequent searches identified two additional YTH proteins: ECT12 of unknown function [26] and CPSF30-L [77]. All these proteins carry canonical core YTH domains, with ECT1 to 11 carrying DF-type domains and ECT12 and CPSF30-L DC-type domains. As in other Viridiplantae, the *Arabidopsis* DF-proteins further categorize into three subclades (DFA, B and C) and the DC-proteins into two subclades (DCA and DCB) [26]. Except for CPSF30, none of the ECT proteins carry other conserved domains outside the YTH, and ECT2 and ECT4 to 8 carry a YPQ-rich region [26].

ECT1 and 2, but not ECT3, 8, 9 or 10, specifically and directly interact, through their YTH domains with the serine/threonine kinase region of CIPK1. Nonetheless, *in vitro* assays showed that CIPK1 does not mediate their phosphorylation [76]. ECT1 was found (in onion epidermal cells) to display a nucleo-cytoplasmic distribution and its YTH domain

proved to be necessary for its nuclear localization. The physiological and molecular roles of ECT1 and other members of the ECT family, except for ECT2, 3 and 4, remain so far unexplored.

In vitro and *in planta* assays showed that ECT2 binds to m⁶A-containing RNAs and requires an intact aromatic cage in its YTH domain, demonstrating that it is an m⁶A-reader [26, 78]. *ECT2* transcript is the most abundant and ubiquitously expressed of all *ECT* genes across plant development, nonetheless, the pattern of expression of its protein is distinct [26, 78, 79]. The highest expression levels of ECT2 are detected two days after seed imbibition, in mature leaves and in whole flowers. It is still detectable at the seedling stage, although to a lesser extent, and is absent in senescent leaves, green and mature siliques and dry and imbibed seeds [26, 79]. ECT2 was also found, as ECT3 to be highly expressed at the site of leaf formation and in emerging leaves what is consistent with their physiological roles [79]. While single *ect2*, *ect3* and *ect4* single loss-of-function mutants display no obvious developmental phenotypes [26, 79], ECT2 and ECT3 act redundantly to ensure the timely emergence and proper leaf development. This physiological role most likely involves ECT2 and ECT3 m⁶A-reading activities since transgenic proteins with a defective aromatic cages fail to restore normal leaf development [79]. Leaf morphogenesis also requires ECT4 but solely in backgrounds where both ECT2 and ECT3 are depleted. The phenotype of the (*ect2;ect3;ect4*) triple mutant is similar, although less penetrant, to that of hypomethylated plants leaves [7]. This suggests that the regulatory function of m⁶A in leaf development and morphogenesis requires decoding by an ECT2/ECT3/ECT4 axis.

Another physiological role of ECT2-m⁶A reading activity relates to trichome morphogenesis [26, 78, 79]. Trichomes are elongated branched single cells found at the surface of the epidermis, in particular of leaf epidermis. They are differentiated protodermal cells that have stopped mitosis and underwent replication cycles without cell division (a process known as endoreduplication). The number of endocycles directly governs the number of branches, with mature trichomes usually carrying three branches, with a DNA content of 32C. Single *ect2* and *ect3* loss-of-function mutants show a significant and similar increase of trichomes with supernumerary branches. This defect is aggravated in the double (*ect2;ect3*) mutant. This cumulative effect suggests that ECT2 and 3 molecular functions in trichome development are not or not fully redundant. Here again transgenic proteins carrying defective m⁶A binding pockets fail to

restore normal trichome development [26, 79] . At least for the single *ect2* mutant, this phenotype is the consequence of extra rounds of endoreduplication occurring in overbranched trichomes [26]. In plants hypomethylated or overexpressing the Arabidopsis homolog of WTAP (FIP37, a core component of the writer complex), trichome morphogenesis is also abnormal, with excess of overbranched trichomes [72, 80]. And similarly to the situation in *ect2*-null mutants, the trichome phenotype of FIP37 overexpressor lines, is the consequence of excessive rounds of endoreduplication cycles [80]. Collectively, these results suggest that, in plants as in animals, m⁶A and at least some of its readers are required for the proper control of cell cycle progression.

The loss of *ect2* induces the rapid degradation and downregulation of at least three trichome-morphogenesis related transcripts that carry m⁶A residues [78]. Nonetheless the precise molecular mechanisms underlying the role of the m⁶A/ECT2 axis in trichome development and how ECT2 and 3 cooperate in this process yet remain to be described.

In planta, ECT2 accumulates both in the nucleus and the cytoplasm, where it shows a diffuse pattern under normal growth conditions [26, 78]. This supports that ECT2 might act in the nucleus at the level of pre-mRNA synthesis and/or maturation and in the cytoplasm where it regulates translation and/or stability. Genome-wide studies suggest that ECT2 might act to stabilize its target transcripts [78] but if and how it controls pre-mRNA processing and whether it also regulates translation is unknown. Upon stress-induced downregulation of translation initiation (heat and osmotic stresses), ECT2 relocalizes to stress granules, which are mRNP triage and storage centers also containing factors of the translation machinery [26, 79]. The formation of cytoplasmic foci upon stress is also a feature of ECT4 but not of ECT3, which is coherent with the presence in ECT2 and 4 (but not in ECT3) of YPQ-rich regions reminiscent of that found in human YTHDF proteins and in aggregation-prone factors.

CPSF30 is a core subunit of the Arabidopsis polyadenylation complex and orthologue to the 30kDa subunit of the Human and yeast Cleavage and Polyadenylation Specific Factor (CPSF). Conversely to other eukaryotes, a conserved feature of the plant genes coding for CPSF30, is that they give rise through alternative splicing and polyadenylation events, to two transcript isoforms (*CPSF30-S* and *CPSF30-L*) [81]. The short form is analogous to other eukaryotic CPSF30 proteins with an array of three characteristic CCCH-type zinc finger motifs (see [82] for a review). The CPSF30-L isoform contains most of the short form polypeptide fused at its C-terminus with a canonical YTH domain

of the DC-type. CPSF30 has both RNA-binding and endonucleolytic activity and regulates the poly(A) site choice of a vast majority of genes, supporting a pivotal role in alternative polyadenylation [82, 83]. Considering CPSF30 involvement in development, programmed cell death, response to plant growth regulators, response to oxidative stress, and role in immunity, this factor was proposed to act as an important integrator of developmental and environmental signals [84]. But all developmental and stress response deficiencies, as well as alterations of poly(A) site choices observed in the loss-of-function mutant were found to be restored to wild-type by the sole expression of CPSF30 short isoform [84], leaving open the question of the role of the CPSF30-L YTH domain.

A distinctive feature of CPSF30-L is that it is localized and retained to the nucleus by itself, while CPSF30-S nuclear localization and retention is dependent upon its association with other subunits of the cleavage and polyadenylation complex (CPSF160 and CPSF73) [85, 86]. This observation allows to suggest that CPSF30-L might exert its role outside of the CPSF complex. Recently, a genetic screen identified CPSF30-L as an important regulator of nitrate uptake and signaling, but also of nitrate accumulation, internal transport and assimilation [85]. A point mutation (Gly to Arg) located in the third zinc-finger domain of CPSF30, affects the plant nitrate regulation network that can be restored to normal by expression of CPSF30-L but not CPSF30-S. This work hence identifies for the first time, one physiological function of CPSF30-L and its YTH domain. CPSF30-L seems to act in this process by controlling the expression of several nitrate responsive genes. In particular it acts upstream of the nitrate sensor NRT1.1 which plays an essential role in nitrate signaling. In the *cpsf30-2* point mutant background, *NRT1.1* levels are reduced and an isoform with a shorter than wild-type 3'-UTR (likely arising from alternative polyadenylation site choice) was found to accumulate. A direct relationship between this abnormal poly(A) site choice and *NRT1.1* mRNA decreased levels yet remain to be demonstrated. Moreover, although it is clear that CPSF30 YTH domain is necessary to fulfill this factor's function in nitrate metabolism, whether this process involves a regulatory role of the m⁶A mark and of CPSF30-L reader function is unknown [85].

2.3.5- The YTH-domain m⁶A reader in *Saccharomyces cerevisiae*

Unlike animals and plants where YTH proteins are present in multiple members, the yeast *Saccharomyces cerevisiae* genome contains only one YTH reader: Pho92 (Phosphate metabolism 92), also known as Mrb1 (Methylated RNA-binding protein 1). Pho92 is an actual m⁶A-reader, as shown by various approaches [87], including the crystallization of its YTH domain in a complex with a 5-mer m⁶A-RNA aptamer. The YTH domain of Pho92 adopts an overall folding that is similar to that of the human YTHDF1 YTH domain; it accommodates the m⁶A residue by forming the conserved binding pocket composed of three aromatic residues and binds methylated oligomers with an affinity comparable to that of human YTH readers [29, 30].

In *S. cerevisiae*, m⁶A deposition is governed by the MIS complex composed of Ime4 (the homolog of METTL3), Mum2 (the homolog of WTAP) and Slz1 and is mostly enriched under meiosis conditions [88, 89]. Like the components of the MIS complex, *PHO92* gene expression is upregulated during meiosis [87, 88] and its deletion delays meiosis entry, albeit less severely than *IME4* deletion does. These observations strongly suggest that Pho92 functions are required for proper meiosis progression and a tempting hypothesis proposes that it acts to regulate meiotic transcripts at the post-transcriptional level. The identification of the direct m⁶A-modified targets of Pho92 during meiosis and the deciphering of how Pho92 influences their post-transcriptional regulation should help understanding the role of RNA methylation decoding in yeast reproductive growth.

In vegetative cells growing in rich media, the deletion of *PHO92* enhances the up-regulation of several genes involved in high-affinity phosphate transport [42], supporting a role for Pho92 in phosphate metabolism. It negatively regulates the stability of *PHO4* mRNA (a key transcription activator in the phosphate signal transduction pathway), through direct binding to a 200 base pair segment in the 3' region of its 3'UTR. Interestingly, the YTH domain alone presents the same binding ability to *PHO4* than the full-length Pho92, supporting that its RNA binding function is mostly carried by the YTH domain. The enhanced degradation of *PHO4* relies to its accelerated deadenylation triggered by the Pho92-mediated recruitment of the Pop2/Ccr4/Not complex likely thanks to an interaction between Pho92 and Pop2 [42]. This feature is reminiscent of the human YTHDF2 reader function, which promotes the decay of m⁶A-modified targets by recruiting the CCR4/NOT complex through direct interaction with the CNOT1 subunit [40]. Intriguingly, introducing YTHDF2 into a *pho92Δ* strain can functionally rescue the accelerated degradation of *PHO4* transcripts

[42] suggesting conserved molecular functions and similar mechanisms of mRNA targeting. *PHO4* does not appear in the recently established list of methylated transcripts under meiosis conditions [87] suggesting either that Pho92 binds non-methylated transcripts or that the *PHO4* mRNA is specifically methylated under non sporulating conditions. Therefore, while Pho92 also promotes mRNA degradation like mammalian YTH readers, the involvement of m⁶A in this pathway as well as direct evidence that this effect requires the m⁶A-reading function of Pho92 are still lacking.

2.4- Regulation of m⁶A-readers expression and function

The expression of m⁶A readers is tightly regulated at the transcriptional and post-transcriptional level. The transcript of human YTHDC1 is differentially accumulated in various rat tissues and alternative isoforms are detected in certain tissues, suggesting the existence of post-transcriptional regulations [23]. In Arabidopsis, while *ECT2* transcript is ubiquitously expressed, its protein is solely present at certain developmental stages [26] suggesting here again the existence of a post-transcriptional control of this gene expression. In yeast, Pho92 is highly regulated in response to external resources. Although present in mitotic cells, Pho92 is strongly induced, as m⁶A-deposition on mRNAs is, when yeasts enter a meiosis program in response to starvation [87, 88]. Conversely, in the presence of high glucose, *PHO92* transcription is repressed by Gcr1, a transcription factor and main regulator of glycolytic genes expression [90]. The tight control of m⁶A-readers expression is crucial at least in Human. Indeed the overexpression of METT3 [3], YTHDF1 [91], DF2 [92, 93] or DC2 [51, 71] has been correlated to proliferation, growth and metastasis formation for several types of cancer. *YTHDF2* up-regulation is due to the downregulation in the context of cancer cells, of two microRNAs that negatively control its transcript accumulation in normal conditions [92, 93]. And *YTHDF1* transcription is induced by the oncogenic c-Myc transcription factor in colorectal cancer cells [91].

In addition, the functions of at least some YTH-domain protein are controlled at the protein level. In *S. cerevisiae*, Pho92 binding to the *PHO4* transcript decreases in low-phosphate conditions, allowing its stabilization and higher protein expression [42]. *In vitro*, binding to *PHO4* 3'-UTR of full length Pho92, but not of its YTH domain alone, is regulated by inorganic phosphate. Since, *in vivo* high phosphate increases Pho92 binding to *PHO4*, this allows to postulate that regions outside the YTH domain are responsible

for phosphate-sensing and regulation of Pho92 RNA-binding property [42]. In plant and Human, the subcellular distributions of several YTH-m⁶A-readers were found to be regulated by cellular and external cues. In the nucleoplasm, YTHDC1 moves from YT-bodies to the nucleoplasm where it binds to insoluble structures when cells enter the G2/M transition of the cell cycle [94]. This relocalization is dependent upon phosphorylation of the protein at yet undetermined position(s) and downregulates YTHDC1 role in alternative splicing events on at least some transcripts [23, 95]. In response to heat stress, YTHDF2 relocates to the nucleus to protect m⁶A residues deposited on the 5'-UTRs of stress-induced genes [15]. In Arabidopsis, ECT1 moves from a nucleocytoplasmic distribution to a nucleoplasmic localization in the presence of elevated calcium concentration. This relocalization depends upon the Ca²⁺-triggered binding of CIPK1 (CalcineurinB-like interacting protein Kinase 1) to ECT1 but does not involve CIPK1-mediated phosphorylation of ECT1 [76]. Calcium is an ubiquitous second messenger, playing pivotal roles in a variety of signal transduction pathways, that allow organisms to respond to developmental and environmental stimuli. ECT1 function is hence likely regulated in response of one or several of these cues. Nonetheless the cellular and molecular implications of this regulatory process remain uncovered so far. In summary, m⁶A readers expression must be precisely regulated and their function is likely to be controlled in response to developmental and/or environmental cues. Nonetheless, whether their m⁶A-reading activities are regulated remains an open question so far.

3- Other m⁶A readers ?

YTH-domain proteins are well-established m⁶A readers, likely to convey m⁶A functions in most organisms and cell types. But the story does not stop there, as it is now clear that the m⁶A mark is decoded by other types of readers. In 2012, Dominissini *et al.* identified (using an untargeted RNA affinity chromatography approach) not only YTH-proteins but also the HuR protein (ELAVL1) as putative readers [96]. Recently, papers by Edupuganti *et al.* [19] and by Arguello *et al.* [18], reported a list of putative non-YTH readers from various types of human and mouse cells (Table 2). They both used quantitative proteomics to detect factors preferentially binding to a methylated bait over an unmethylated control. These studies identified thirty-three putative new m⁶A readers, amongst which nineteen are known RNA binding proteins and five carry zinc finger

domains and could hence bind RNA. The remaining proteins carry various functions not obviously connected to RNA. Without additional experiments, it is difficult to discriminate actual readers from aspecific interactants, such as PRMT10 (an arginine methyl-transferase that could be retained by the methyl group) or from effector proteins indirectly recruited to the bait. The XRN1 5'-3' exoribonuclease, identified in the Edupuganti screen, was for example found to be recruited as effector by YTHDF2 and the HuR protein could be an effector of the non-YTH reader IGF2BP1 [97]. Nonetheless Arguello and colleagues, by photo-crosslinking the cell crude extract to the affinity column, likely captured proteins in direct contact with the RNA bait. They identified five proteins, including LRPPRC and FMR1 (Fragile-X Mental Retardation 1) for which they subsequently demonstrated a direct and preferential binding to m⁶A-modified RNAs, supporting that they are actual readers [18].

Up-to-date studies by Edupuganti [19], Arguello [18] and others [3, 14, 21, 22, 97, 98] allowed the identification of seven non-YTH m⁶A readers. Strikingly, these proteins convey the molecular roles of m⁶A on pre-mRNA splicing, mRNA stability, translation and storage control, as YTH readers do. In the nucleus, three members of the Heterogeneous nuclear RiboNucleoProtein family (HnRNP): HNRNPC, G and A2B1 were characterized as actual m⁶A readers [21, 22, 98]. HNRNPC and G were firstly identified as proteins that preferentially bind m⁶A-modified RNAs through an "m⁶A-switch" mechanism in which the m⁶A-mediated destabilization of an RNA hairpin exposes their RNA binding sites [21, 22]. *In cellulo*, they act to convey the m⁶A signal of a subset of m⁶A-dependent alternative splicing events but they are likely to work separately, as their m⁶A-modified RNA targets are distinct [21]. Conversely to HNRNPC and G, HNRNPA2B1 recognizes its m⁶A-modified targets not through an m⁶A-switch mechanism but through direct recognition of the methylated RGAC consensus site by a yet unknown mechanism [98]. HNRNPA2B1 binds some five hundred nuclear mRNAs in an m⁶A-dependent manner and regulates a subset of m⁶A-controlled alternative splicing events. HNRNPA2B1 also conveys the role of m⁶A on microRNA (miRNA) biogenesis, by recruiting the microprocessor complex to the pri-miRNA. It controls the accumulation of 50% of the miRNAs, which carry m⁶A modifications, suggesting than other reader(s) conveying m⁶A roles on these small RNAs might exist [98].

In the cytoplasm, four true m⁶A-readers have been identified. The translation initiation complex eIF3 [14], the METTL3 methylase [3] and FMR1 [18, 19] convey m⁶A signals on

translation and IGF2BP1/2/3 (Insulin-like Growth Factor 2 Binding Protein) on mRNA stability and storage control [97]. FMR1 negatively regulates the translation of a subset of m⁶A-modified transcripts, acting downstream to the m⁶A signal [19]. METTL3 is the methylase subunit of m⁶A writer complex, which localizes in the nucleus [3]. METTL3 is recruited to m⁶A marks in the 3'-UTRs of a pool of transcripts to promote their translation by recruiting the eIF3 initiation complex. It acts in this process independently from other components of the writer complex and does not require its catalytic activity and consistently with a role in translation, a subset of METTL3 was found to be present in the cytoplasm [3]. The proof of METTL3 ability to directly accommodate the m⁶A residue is still lacking but tethering assays by Lin et al. [3], demonstrate that it is part of a yet unidentified reader complex, if not being itself a reader of the mark. In the context of heat stress, eIF3 binds to the m⁶A residues added to the 5'-UTRs of stress responsive genes, such as chaperone genes, and permits their expression through a cap-independent mechanism. The m⁶A-eIF3 axis hence allows the expression of stress proteins in the context of the general heat-induced inhibition of the eIF4F-dependent translation initiation process [14]. The IGF2BP proteins were recently identified as new m⁶A-readers, which preferentially bind methylated transcripts at the UGGAC motif. IGF2BP1, and putatively BP2 and 3, acts to recruit stabilizers amongst which HuR, to a subset of its targets, stabilizing them in an m⁶A-dependent manner [97]. Moreover, IGF2BP1 relocates into mRNP aggregates of the Stress Granule type, following heat stress, suggesting that it might also take part to the storage of translationally inactive mRNAs under stress conditions, hereby contributing to prevent their degradation [97].

How these non-YTH readers recognize the m⁶A-residues remains to be elucidated at the molecular level. The translation initiation factor eIF3 is a multisubunit complex likely binding m⁶A through a multifaceted surface that yet remains to characterize [14]. The four K-Homology (KH) domains of IGF2BPs were found to cooperate in the binding to a single-stranded(ss)-m⁶A probe, with the KH3 and 4 being indispensable for binding [97]. Moreover, the three KH domains of FMR1, together with its RGG repeat motif, were found necessary to bind ss-m⁶A RNAs *in vitro* [19]. Since several of the putative readers identified through untargeted approaches (Table 2), carry KH domain, it suggests that these evolutionarily conserved and structured domains might contribute, alone or together with other domains, in binding to m⁶A residues.

The discovery of additional m⁶A-reader, add further ways for the m⁶A mark to regulate gene expression. This variety of readers, many of which likely remain to be identified, open wide possibilities to fine-tune cell gene expression patterns. Moreover, distinct readers are likely to interplay in the regulation of common targets, further adding possible layers of regulation. FMR1 for example negatively regulates in an m⁶A-dependent manner the translation of its targets, a role in contrast with the global positive role of m⁶A on translation. Nonetheless, 80% of the transcripts directly bound by YTHDF1, are also targets of FMR1, supporting that, at least on a fraction of m⁶A-controlled translation events, this mark can be at the center of a complex interplay of readers that permit the positive or negative fine-tuning of their translation according to the transcript context.

4- Conclusive remarks

Since the first identification in 2012 [96], m⁶A readers proved to be fascinating RNA binding proteins crucial to maintain proper cellular activity, organism reproduction, growth and development. Recently, the demonstration of the existence of non-YTH readers in mammals, added new possible ways to convey the m⁶A signal. Conversely to YTH-domain protein, their recognition and the comprehension of their role as readers is still in its infancy. How these non-YTH proteins recognize the m⁶A-residu? Do some of them carry methyl binding pockets, or does the presence of the modified residue simply increase their affinity without accommodating directly de methyl moiety? What are the molecular and physiological implications of their m⁶A-reading activities ?

In organisms other than mammals, identification and study of m⁶A readers is much less advanced. Yet, for example, the dramatic effect of the loss of m⁶A in thale cress, strongly suggests that they have also important functions in plants. Other types of readers are likely to exist outside mammals, as supported for example by the observation that the presence of m⁶A is not always accompanied by the existence of YTH-domain proteins in different eukaryote lineages. Their identification is an important step to get a more complete understanding of the regulatory roles of the m⁶A mark.

At the moment, the picture emerging is that m⁶A signaling is fundamental at transitions, when following developmental and most likely environmental cues, cells must change identity or activity to grow or acclimate. This regulatory potential putatively relies on the dynamic nature of this mark, which can be erased, but also on its substoichiometric

distribution, as not all transcripts carry m⁶A and the whole pool of a given transcript might not be homogeneously m⁶A-modified. But this regulatory potential also leverages on the decoding capabilities of readers.

The genomes of most eukaryotes code for multiple members of the YTH-domain protein family and one can anticipate that other types of readers, yet largely remaining to be identified and studied, convey the m⁶A signal. Interplays between readers are likely to exist. Readers can compete for a same m⁶A residue, as it seems to be the case for YTHDF1 and DF2, which appear to act antagonistically respectively promoting translation and decay, on a same set of transcripts. Readers can also cooperate; YTHDF3 hands over transcripts to DF1 or DF2 in HeLa cells, suggesting that it participates to the process of mRNA selection by other readers. In *Arabidopsis thaliana*, ECT2 and 3 are both separately required for trichome morphogenesis and their joint loss of function aggravates trichome developmental defects, arguing against a full redundancy at least in this specific developmental process. There also seems to exist interplays between YTH and non-YTH readers. YTHDF1 and FMR1 share a pool of target transcripts which translation they regulate antagonistically. The diversity of readers carried by most organisms hence pledges vast possibilities of combinations where, depending on the type and stoichiometry of bound reader(s), the fate and/or expression of a given transcript will be differentially controlled. Uncovering the interplays that exist between the different readers expressed in cells in a given context and how this interplay is modified to answer developmental or environmental stimuli, is one of the next challenges of the m⁶A reader field.

Regulating readers expression and function is an additional powerful mean to ensure plasticity by modifying m⁶A decoding capabilities and hence quickly fine-tune cell gene expression pattern. A precise timely control of YTH readers expression in proper amount is crucial. Misregulation of YTH readers expression can be deleterious in mammalian cells as it correlates with cancer development and poor patient prognosis. In *Arabidopsis*, the dichotomy between ECT2 transcript and protein accumulation across development supports a precise post-transcriptional regulation that might be important for plant development. And of course regulating readers to alter their subcellular distribution, the type of effectors they recruit, or even their RNA binding property/m⁶A binding ability, is another way to contribute to the regulatory and dynamic roles of the m⁶A mark. The existence of such regulatory processes in response

to diverse stimuli has been observed in mammals, budding yeast or thale cress (see chapter 2.4). The usual suspects to orchestrate these regulations are post-translational modifications, such as phosphorylations, as it is the case for YTHDC1 cellular localization that is controlled through phosphorylation during cell cycle progression. Understanding how different readers are regulated at various developmental and/or stress-related transitions is also critical to elucidate the full spectrum of m⁶A influences on gene expression.

Acknowledgements

The work on plant YTH-readers was primarily supported by the CNRS, the University of Perpignan (UPVD), the Institut Universitaire de France (IUF) and l'Agence Nationale pour la Recherche (ANR HEAT-EpiRNA: ANR-17-CE20-0007-01 and ANR HEAT-Adapt: ANR-14-CE10-0015). Soizik Berlivet is the recipient of a post-doctoral position financed by the ANR through contract ANR 3'ModRN: ANR-15-CE12-0008-12. This work was also supported by networking activities in the frame of the COST program EpiTran (CA16120, <https://epitrان.eu/>) and by the Labex TULIP.

Function	Name	Organism	Mutant Phenotype	Ref.
Neuronal functions	YTHDF1	mouse	Default in axon regeneration	[48]
	YTHDC1 (YT531-B)	fruit fly	Behavior defects	[5, 35, 49]
Sex Determination	YTHDC1	mouse	Default in XIST-mediated gene silencing	[52]
	YTHDC1 (YT531-B)	fruit fly	Decrease female viability and sexual transformation	[5, 6, 35]
Germline maturation	YTHDC2	mouse	Failure to execute meiotic Prophase I	[58]
Cell Differentiation	YTHDF2	mouse & zebrafish	Failure to differentiate	[53, 54]
Stress Response	YTHDF2	mammalian cell line	Reduction of 5'-UTR heat-stress induced methylation	[15]
	YTHDF3		circRNA translation increase	[44]
	Pho92	<i>S. cerevisiae</i>	In poor medium conditions that induce sporulation, <i>pho92-Δ</i> yeasts are delayed in meiosis entry	
Nutrient uptake	Pho92	<i>S. cerevisiae</i>	Involved in Pi Uptake Higher cellular Pi concentration in <i>pho92-Δ</i> yeast	[42]
	CPSF30-L	<i>A. thaliana</i>	Involved in Nitrate uptake, accumulation, transport and assimilation by the plant Mutant plants have lower nitrate content in the roots and shoots	[85]
Leaf Morphogenesis	ECT2 ECT3 ECT4	<i>A. thaliana</i>	No defect of single <i>ect2</i> , <i>ect3</i> or <i>ect4</i> loss-of-function mutants (<i>ect2;ect3</i>) mutant shows a delay in the emergence of the 1 st true leave and serrated, triangular shape leaves No defect for (<i>ect2;ect4</i>) or (<i>ect3;ect4</i>) double mutant but the (<i>ect2;ect3;ect4</i>) triple mutant shows an aggravation of (<i>ect2;ect3</i>) phenotypes	[79]
Trichome morphogenesis	ECT2 ECT3	<i>A. thaliana</i>	- Single mutants have higher levels of overbranched trichomes -Double mutant have even higher levels of overbranched trichomes - Single <i>ect2</i> mutant have increased ploidy in trichomes	[26, 78, 79]

Table 1: Physiological and cellular roles of YTH m⁶A readers

Name	Cell type	Function / Conserved Domains
RBBP6	HeLa (N) mESC (C)	E3 ubiquitin-protein ligase. Involved in YBX1 and ZBTB38 ubiquitination and proteasomal degradation 1 CCHC-type zinc finger / 1 RING-type zinc finger / 1 DWN domain
CPSF6	HeLa (N)	Part of the Cleavage Factor Im complex. Binds to cleavage and polyadenylation substrates, promotes RNA looping. 1 RRM
HNRNPH1 HNRNPH2 HNRNPF	HeLa (N)	Components of heterogeneous nuclear ribonucleoprotein (hnRNP) complexes, which provide the substrate for the processing events that pre-mRNAs undergo before becoming functional. 3 RRMs / 1 putative zinc finger
TARDBP	HeLa (N) mNPC (N)	DNA & RNA-binding protein. Regulates transcription, pre-mRNA splicing, mRNA stability. 2 RRMs
KHSRP/ FUBP2	HeLa (N) mESC (N) mNPC (N) mouse 3T3	Involved in: (i) tissue-specific alternative splicing events, (ii) degradation of unstable mRNA with ARE elements. May play a role in mRNA trafficking. May interact with ssDNA. 4 KH domains
FUBP3	HeLa (N) mouse 3T3	May interact with ssDNA from the far-upstream element (FUSE). May activate gene expression. Same family as KHSRP 4 KH domains
FMR1^{a,b}	HeLa(N, C, T) mESC (C)	Polysome-associated RBP. Regulation of alternative splicing, mRNA stability, mRNA transport, localized translation. 2 Agenet-Like domains / 3 KH domains / 1 RGG motif
FRX1 FRX2	HeLa (N, C) mESC (C)	FR agile X related 1 & 2 : Autosomal homologs of the fragile X mental retardation gene 1 Agenet-like domain / 2 KH domains
IGF2BP3^c	HeLa (N)	Insulin like Growth Factor 2 mRNA Binding protein 3 . May recruit transcripts to cytoplasmic mRNPs. Binds to mRNAs:(i) 3'-UTR of CD44 (stabilizes it), (ii) ACTB and MYC and (iii) 5'-UTR of IGF2 2 RRMs / 4 KH domains
COLGALT1	HeLa (N)	Beta-galactosyltransferase: transfers beta-galactose to hydroxylysine residues of type I collagen. ER-Retention Motif
CNBP	HeLa(N, C) mESC (C) mouse 3T3	ssDNA-binding protein, with specificity to the sterol regulatory element (SRE). 7 CCHC-type zinc fingers
ZCCHC8	HeLa (C) mESC (C)	Scaffolding subunit of the nuclear exosome-targeting (NEXT) complex. 1 CCHC-type zinc finger
XRN1	HeLa (C)	Major 5'-3' exoribonuclease involved in mRNA decay.
SF3B4	HeLa (C) mESC (C)	Splicing Factor 3B subunit 4 : Involved in pre-mRNA splicing as a component of the splicing factor SF3B complex 2 RRMs
LRPPRC^b	HeLa (T) mESC (N)	Binds to HNRNPA1-associated mRNAs. Part of nuclear mRNPs. Binds to mitochondrial poly(A) mRNAs. May be involved in nuclear transcription. Role in translation or stability of mitochondrially encoded cytochrome c oxidase. PPR Repeats
TIMM8B	mESC (C)	Translocase of Inner Mitochondrial Membrane 8 homolog B : Chaperone-like that protects the hydrophobic precursors from aggregation and guide them through the mitochondrial intermembrane space Twin-CXC3 motif
SRSF7	mESC (C) mouse 3T3	Serine and arginine rich splicing factor: Required for pre-mRNA splicing. Put. Adapter for mRNA nuclear export. 1 CCHC-type zinc finger
ZNFX1	mESC (C) mouse 3T3	Zinc finger NFX1 -type containing 1 6 NFX1-type zinc fingers
PRMT10	mESC (C)	Arginine methyltransferase. Mediates the symmetrical dimethylation of SF3B2. Regulation of alternative splicing 2 TPR repeats / AdoMet-MTase type I motif
RBM7	mESC (C)	Subunit of the trimeric nuclear exosome-targeting (NEXT) complex. 1 RRM / 2 CZZHC8-type zinc fingers
L1TD1	mESC (C)	LINE1 type transposase domain containing 1 1 Coiled-Coil domain
DIS3/ RRP44	mouse 3T3	Putative catalytic component of the RNA exosome complex which has 3'->5' exoribonuclease activity 1 PINc Domain
ERCC2	mouse 3T3	ATP-dependent 5'-3' DNA helicase, component of the transcription and DNA repair factor IIH (TFIIH) core complex 1 Helicase ATP-Binding domain / 1 DEAD-box domain
SP140	mouse 3T3	Component of the nuclear body, also known as nuclear domain 10, PML oncogenic domain, and KR body 1 PHD-type zinc finger
HSD17B12	mouse 3T3	hydroxysteroid 17-beta dehydrogenase 12 : enzyme involved in the long-chain fatty acids elongation cycle
SLC4A1AP/ Kanadaplin	mouse 3T3	Putatively involved in the targeting of kAE1 vesicles (through direct binding to the kAE1 protein) to their final destination. 1 FHA (forkhead-associated) , 1 coiled coil, 1 Glu-Rich region
PSMA6	HeLa (T)	Component of the 20S core proteasome complex
HBA1	HeLa (T)	Hemoglobin subunit alpha 1 Hb-alpha-Like domain
SAMHD1	HeLa (T)	Host restriction ribonuclease activity and is involved in defense response to virus. 1 SAM Domain / 1HD domain (Zn ²⁺ binding site)
HuR	HepG2 (T)	Involved in the stability and export control through direct binding of UTRs of its target RNAs. Binds to the 3'-UTR region of mRNAs and increases their stability. Binds to poly-U elements and AU-rich elements (AREs) in the 3'-UTR of target mRNAs. Preferentially binds mRNAs without m6A marks, stabilizing them, promoting ESCs differentiation (By similarity). 3 RRMs

Table 2: List of putative non-YTH m⁶A readers identified by untargeted quantitative proteomics. Data were retrieved from [18] and [19]. Work by [19]

identified m⁶A readers from nuclear and/or cytoplasm of various cell types (HeLa, mESC, mNPC) and from nuclear extracts of mouse 3T3 cells, work by [18] identified m⁶A readers from total extracts of HeLa cells and work by Dominissini et al. from total HepG2 cell extracts (ref). Raw number 2 reports the cell type(s) from which the m⁶A reader has been identified: N is for nuclear, C from cytoplasmic and T for total extract. Raw number 3 reports a brief description of the function of the protein and the list of conserved domains it carries. Information about protein functions was retrieved from HGNC at <https://www.genenames.org/> and conserved domain architecture from Uniprot (<http://www.uniprot.org>) or InterPro (<https://www.ebi.ac.uk/interpro/protein/>). Proteins labeled in red were confirmed as actual m⁶A-readers by direct approaches in papers: a [19], b [18] or c [97].

Figure Legends

Figure 1: m⁶A molecular impact on RNA. (A) m⁶A acts to repel RBPs or to attract so called readers: RBPs that directly bind the m⁶A thanks to their YTH domain or through yet unknown molecular mechanisms [19]. m⁶A-readers in turn recruit effectors to the transcript and/or carry themselves effector domains that will control mRNA fate. (B, C) m⁶A acts as a molecular switch. (B) If the m⁶A-residue is part of the RNA structure, it destabilizes the RNA duplex [20]. In the RNA hairpin of the lncRNA MALAT1, this m⁶A-mediated structural rearrangement predisposes to RBP binding [17]. (C) If the m⁶A-residue is located immediately 5' or 3' to the structure, it stabilizes the RNA duplex ([20]). In this latter case, readers binding these m⁶A-stabilized structures are yet to be identified.

Figure 2: Evolutionary history of YTH proteins in Eukaryotes. (A) The presence and organization of YTHDC and YTHDF proteins in major eukaryotic lineages is schematically represented. YTHDF type is represented in green, the YTHDC type in blue and extra functional domains fused to YTH in red. NF: not found in the available genomic data. (B) Alignment of 16 YTH domains representative of the diversity illustrated in (A). The origin of the sequence, its YTH types and the nature of the extra functional domain fused to the YTH, when present, is indicated on the left side of the alignment. The position of the putative secondary structure elements (predicted using the PHYRE2 program www.sbg.bio.ic.ac.uk/~phyre2/) is represented by arrows above the

alignment. The three amino acids essential for m⁶A binding are marked by asterisks. The intensity of the blue color is representative of the level of conservation of each position.

Figure 3: Evolutionary history of YTH proteins in the Viridiplantae lineage. (A) A gene coding for a YTHDF protein was likely present in the common ancestor of Viridiplantae but lost in the Chlorophyte lineage. In the common ancestor of angiosperms, a gene duplication event, likely followed by the neofunctionalization of the generated copies, gave rise to the FA, FB and FC subgroups, present in all modern angiosperm species. **B)** A single gene coding for YTHDC protein was likely present in the common ancestor of Viridiplantae as well as an independent gene coding for a zinc fingers protein. For Chlorophyte, this *YTHDC* gene was lost in the *Micromonas* and *Ostreococcus* lineage but conserved in the *Chlamydomonas* and *Volvox* lineages. In the common ancestor of Embryophyte, two YTHDC versions (DC1 and DC2) evolved from this ancestral YTHDC sequence. In the DC1 version, the YTH domain was fused to the zinc fingers domain that ceased to be coded independently from YTH in most plants. Subsequently, YTHDC2 was lost in the monocot lineage, while conserved in the eudicot lineage (*: with the exception of three Fabidae species, *Cucumis sativus*, *Medicago truncatula* and *Trifolium pratense*, and the eudicot outgroup species *Aquilegia coerulea* where the DC2 version is also apparently absent).

Figure 4: Molecular roles of animal YTH domain proteins in transducing the m⁶A signal. The nuclear YTHDC1 protein promotes splicing of specific pre-mRNAs through recruitment of the phosphorylated form of SRSF3 or of SRSF10, each protein competing for YTHDC1 binding. YTHDC1 can also promote RNA nuclear export when it recruits the hypophosphorylated form of SRSF3 as effector. The molecular roles of YTHDC2 are still unclear and may involve the recruitment of effector partners. The cytoplasmic m⁶A reader YTHDF1 promotes translation of its target. YTHDF3 allows better specificity of YTHDF1 and DF2 binding to their target RNAs. YTHDF2 can recruit the CCR4-NOT complex to initiate deadenylation and subsequently mRNA degradation. Cytoplasmic and nuclear YTH reader proteins are schematically represented in blue and orange, respectively. Effector proteins are represented in green.

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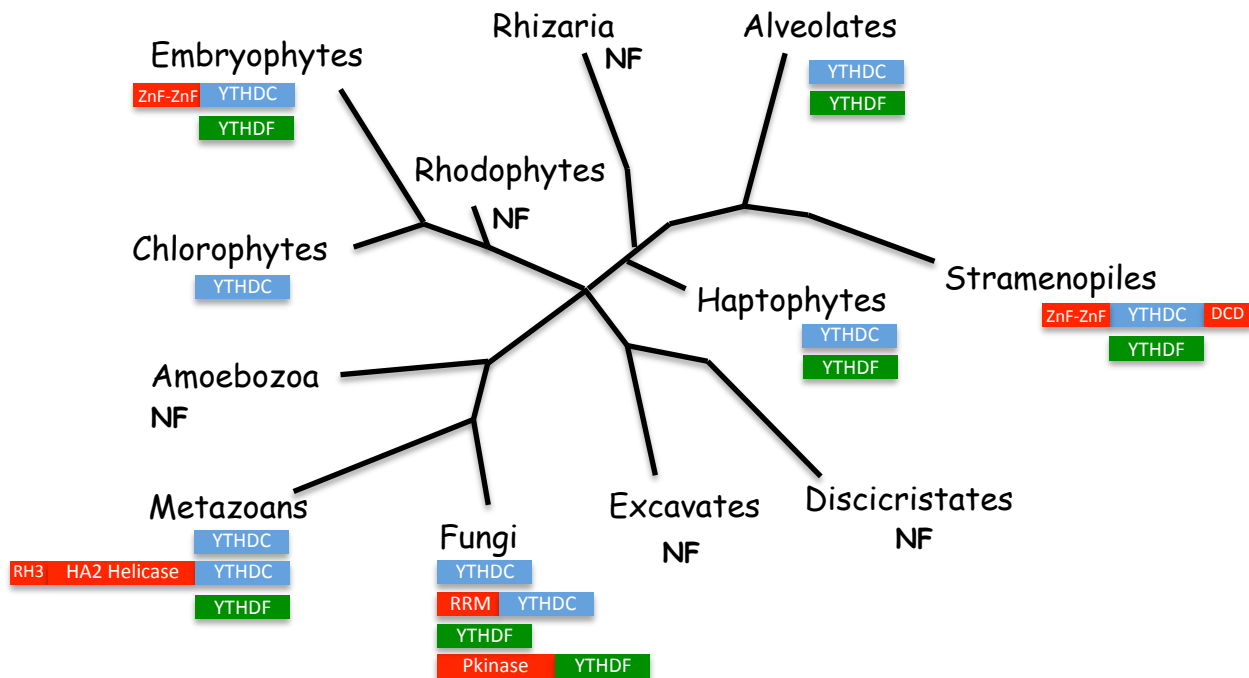
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A



B

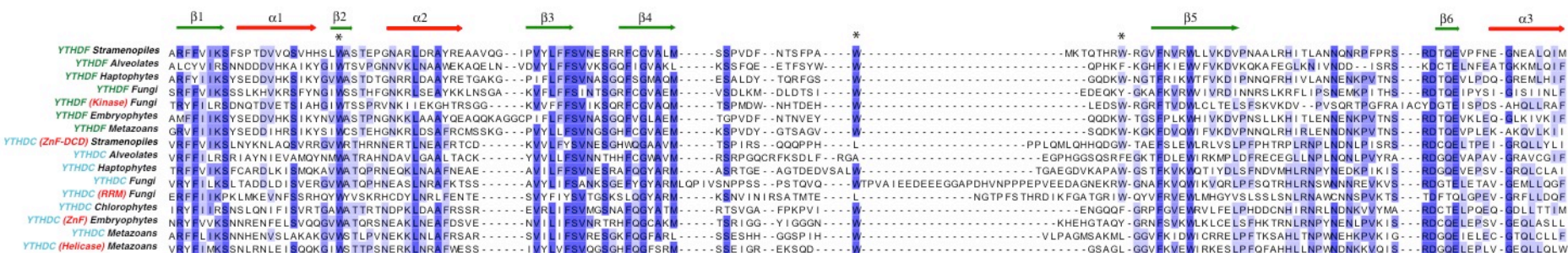


Figure 2
Berlivet et al., BBA

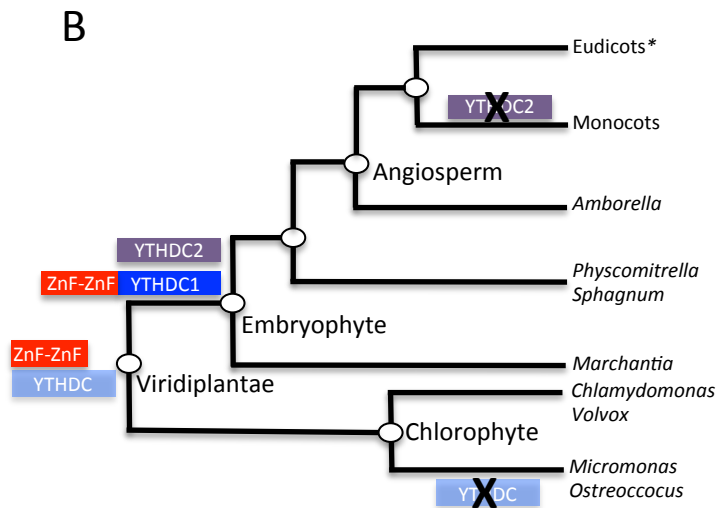
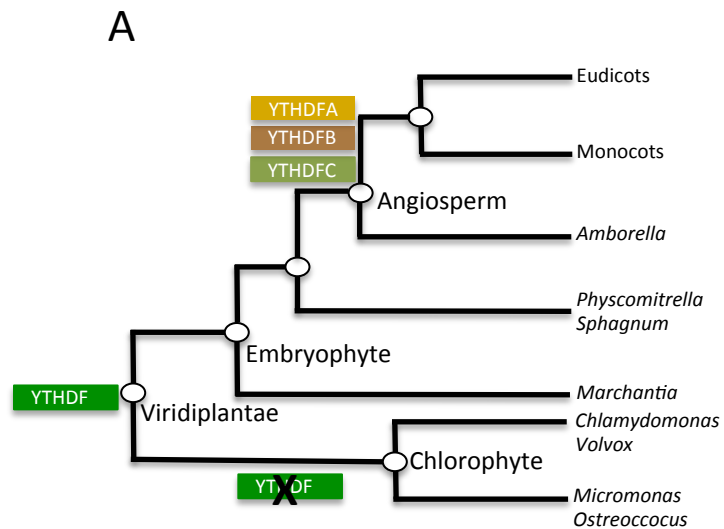


Figure 3
Berlivet et al. BBA

