

Identification of putative reader proteins of 5-methylcytosine and its derivatives in *Caenorhabditis elegans* RNA

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SHORT TITLE (48 characters)

Readers of m⁵C and derivatives in *C. elegans* RNA

KEYWORDS

RNA, 5-methylcytosine, *Caenorhabditis elegans*, epitranscriptomics, readers

ABSTRACT (147 words)

Methylation of carbon-5 of cytosines (m^5C) is a post-transcriptional nucleotide modification of RNA with widespread distribution across organisms. m^5C has been shown to participate in mRNA transport and maintain mRNA stability through its recognition by the reader proteins ALYREF and YBX1, respectively. We recently showed that m^5C is required for *Caenorhabditis elegans* development and fertility under heat stress. To contribute to the understanding of how m^5C and its oxidative derivatives mediate their functions, we developed RNA baits bearing modified cytosines in diverse structural contexts to pulldown potential readers in *C. elegans*. Our mass spectrometry analyses reveal unique binding proteins for each of the modifications. We validate our dataset by demonstrating that the nematode ALYREF homologues ALY-1 and ALY-2 preferentially bind m^5C *in vitro*. The dataset presented here serves as an important scientific resource that will support the discovery of new functions of m^5C and its derivatives.

INTRODUCTION

Nucleoside chemical modifications are a common feature in RNA molecules. More than 160 post-transcriptional modifications have been reported since the discovery of pseudouridine in 1957 (Davis & Allen, 1957; Cohn, 1960; Boccaletto *et al*, 2017). However, the molecular and physiological functions of most RNA modifications remain unknown.

Discovered in 1958 (Amos & Korn, 1958), the methylation of carbon-5 of cytosines in RNAs (m^5C) is now known to be a conserved and widely distributed feature in biological systems. m^5C is catalysed by tRNA aspartic acid MTase 1 (DNMT2) and RNA methyltransferases from the Nop2/Sun domain family (NSUN1-7 in humans), and has been shown to be involved in tRNA stability, ribosome fidelity and translation efficiency (reviewed in García-Vílchez *et al*,

2019). Previous studies showed that m⁵C is subject to hydroxylation and oxidation by alpha-ketoglutarate-dependent dioxygenase ABH (ALKBH1), forming 5-hydroxymethylcytidine (hm⁵C) and 5-formylcytidine (f⁵C) (Huber *et al*, 2015; Kawarada *et al*, 2017). Further processing can occur through 2'-O methylation by FtsJ RNA Methyltransferase Homolog 1 (FTSJ1), yielding 2'-O-methyl-5-hydroxymethylcytidine (hm⁵Cm) and 2'-O-methyl-5-formylcytidine (f⁵Cm) (País de Barros *et al*, 1996; Huber *et al*, 2017; Kawarada *et al*, 2017). While the biogenesis of these chemical marks has been elucidated (Navarro *et al*, 2020; Kawarada *et al*, 2017), their functions remain largely unexplored.

The identification of readers, *i.e.* proteins that specifically, or preferentially, interact with RNA molecules bearing a certain modification, has proven to be an important step towards the understanding of a modification's biological function. For example, the discovery of N⁶-methyladenosine (m⁶A) readers added new layers of complexity to this pathway, revealing context- and stimuli-dependent functions previously not appreciated (Shi *et al*, 2019). To date, only two reader proteins have been identified for m⁵C. The Aly/REF export factor (ALYREF) was identified in an RNA pulldown experiment and shown to interact with NSUN2-modified CG-rich regions and regions immediately downstream of translation initiation sites. NSUN2-mediated mRNA methylation is interpreted by binding of ALYREF to modulate nuclear-cytoplasm shuttling of transcripts. (Yang *et al*, 2017). More recently, the Y-box binding protein 1 (YBX1) was shown to recognise m⁵C in mRNAs and enhance its stability through the recruitment of ELAV1 in human urothelial carcinoma cells (Chen *et al*, 2019). In zebrafish, YBX1 was shown to stabilise m⁵C-modified transcripts via Pabpc1a during the maternal-to-zygotic transition (Yang *et al*, 2019).

Recently, we used *Caenorhabditis elegans* as a model to engineer the first organism completely devoid of m⁵C in RNA. We mapped m⁵C with single nucleotide resolution in different RNA species and found that m⁵C is required for physiological adaptation and translation efficiency

at high temperatures (Navarro *et al*, 2020). Here, to further expand our knowledge on the m⁵C pathway in this organism, we produced the first list of putative readers of m⁵C and its metabolic derivatives hm⁵C, hm⁵Cm and f⁵C. The putative readers are enriched in proteins with roles in germline development and RNA surveillance. We show that the *C. elegans* ALYREF homologues ALY-1 and ALY-2 preferentially bind m⁵C *in vitro* and identify other proteins that contain a conserved RNA recognition motif potentially involved in m⁵C binding.

RESULTS

To increase the chances to identify *bona-fide* modification readers, we used modified RNA baits with biologically relevant characteristics. Considering that m⁵C is deposited in tRNAs in a structure-dependent manner, we designed baits bearing modifications in three structural contexts: single-strand, double-strand, and loop (**Figure 1A**). m⁵C, hm⁵C, hm⁵Cm and f⁵C monomers were synthesised and incorporated into oligonucleotides tethered to triethylene glycol (TEG)-biotin, as previously described (Tanpure & Balasubramanian, 2017).

The RNA pulldown experiments were performed in independent biological triplicate. We incubated the RNA baits with pre-cleared whole worm lysates prepared from synchronised populations of gravid adults. To increase the stringency of our screen, we included two controls: beads only and an unmodified RNA bait. Following the pulldown experiments, proteins were eluted from the RNA baits and identified by mass spectrometry.

Proteins were ranked according to their enrichment in modified RNA pulldowns in comparison to the controls. Proteins were considered candidate readers if they preferentially bound to the modified-RNA bait, i.e., at least 2.0 and 1.5 fold increased as compared with the beads and unmodified C controls, respectively (**Figure 1B**).

To narrow down the number of candidate readers, we focused on unique binders, *i.e.* proteins that bound specifically to a single modified RNA bait. Using these criteria, we found 143, 62, 81 and 33 putative unique readers for m⁵C, hm⁵C, hm⁵Cm and f⁵C, respectively (**Figure 2A**, **Supplemental Table S1**). We used the WormBase Enrichment Suite to perform phenotype enrichment analysis on the lists of unique candidates (Angeles-Albores *et al*, 2016). This tool analyses a list of genes according to phenotypes that have been reported by researchers to WormBase upon knockout or RNAi, thus suggesting a potential involvement of such genes in specific biological processes. m⁵C unique binders are the most abundant proteins when compared to its oxidised derivatives (Huber *et al*, 2015, 2017), likely due to higher levels of this modification in RNA. This subset is enriched mostly for germline development phenotypes, and a few RNA surveillance processes. hm⁵Cm-unique binders show specific enrichment for RNA processing phenotypes, whereas hm⁵C and f⁵C show exclusively germline-related phenotypes (**Figure 2B**).

To validate our dataset, we verified whether ALYREF homologues could be found among our ranked m⁵C reader candidates. Yang *et al*. identified amino acid residues on ALYREF required for binding to m⁵C (Yang *et al*, 2019). Protein alignments show that some of these residues are conserved in ALY-1 and ALY-2 (**Figure 3A**). Indeed, ALY-1 and ALY-2 are 1.8-fold enriched across all triplicates of m⁵C pulldowns (**Supplemental Table S1**). We used transgenic strains expressing either ALY-1 (OP502), or ALY-2 (OP217), tagged at the C-terminus with TY1::eGFP::3xFLAG, to confirm these findings (Sarov *et al*, 2012). We performed pulldown experiments under the same conditions as before and confirmed using western blotting that both ALY-1 and ALY-2 preferentially bind m⁵C *in vitro* (**Figure 3B**).

Some m⁶A readers use common RNA binding domains to identify modified RNA (Shi *et al*, 2019). Amino acid residues shown to be important for m⁵C binding in ALYREF also reside in the RNA recognition motif (RMM) domain, which is a known RNA-binding domain (Yang *et*

al, 2017). The *C. elegans* orthologues ALY-1 and -2 are predicted to have a similar protein fold in the same region (**Figure 3C**).

Having shown that ALY-1 and ALY-2 preferentially bind m⁵C-modified RNA, we wondered if other RRM-containing proteins were also identified as m⁵C unique binders. We found that three other proteins with one or more RRMs, RNP-4, SQD-1 and PAB-2, bind to m⁵C baits preferentially. RRM domains consist of a four-stranded antiparallel β -sheet with two α -helices packed against it (Maris *et al*, 2005). Sequence alignments of the RRM domains in ALY-1, ALY-2, RNP-4, SQD-1, PAB-2 and ALYREF show that a high degree of conservation exists across these proteins in the two ribonucleoprotein (RNP) consensus sequences that are important for nucleic acid binding. Interestingly, a phenylalanine residue outside of the two RNPs is highly conserved in all six proteins (**Figure 3D**).

In summary, we have identified putative readers for m⁵C, hm⁵C, hm⁵Cm and f⁵C in *C. elegans* using proteomics. To verify the relevance of this proteomic dataset, we confirmed that ALY-1 and ALY-2 interact with m⁵C orthogonally. Finally, we identified three candidate proteins that share a conserved RRM domain with ALY-1/2 (RNP-4, SQD-1 and PAB-2), highlighting these as potential *bona fide* m⁵C readers.

DISCUSSION

This work presents a list of candidate readers of the m⁵C oxidative derivatives hm⁵C, hm⁵Cm and f⁵C. Stable isotope labelling experiments suggest that hm⁵Cm is a stable modification (Huber *et al*, 2017). In addition, it has been shown that the ratios between hm⁵C, hm⁵Cm and f⁵Cm in the tRNA Leu-CAA pool varies between organisms and tissues, suggesting a dynamic regulation of these marks in different physiological contexts (Kawarada *et al*, 2017). This could suggest that these modifications carry out cellular functions themselves, rather than being

metabolic by-products of a demethylation pathway. Considering the reported function of f⁵C in mitochondrial protein synthesis (Takemoto *et al*, 2009), it is expected that the oxidation of m⁵C in tRNAs might affect their role in cytoplasmic translation. The identification of readers provides a first step to elucidate these functions. Importantly, pathogenic mutations have been mapped to NSUN2, NSUN3 and FTSJ1, suggesting that imbalances in these modifications might contribute to the pathogenesis of human diseases (Abbasi-Moheb *et al*, 2012; Khan *et al*, 2012; Komara *et al*, 2015; Van Haute *et al*, 2016; Guy *et al*, 2015; Takano *et al*, 2008; Ramser *et al*, 2004; Freude *et al*, 2004).

The use of synthetic modified RNAs as a bait for identification of proteins that are either specifically, or preferentially, interacting with ribonucleoside modifications has been applied with success in the past (Dominissini *et al*, 2012; Yang *et al*, 2017; Chen *et al*, 2019; Yang *et al*, 2019). Rather than focusing on sequence context, we developed baits bearing modifications in diverse structural contexts. In this work, we identified two proteins showing higher binding affinity to modified RNA: ALY-1 and ALY-2.

The genome of *C. elegans* encodes three members of the Aly/REF family. It has been shown that simultaneous downregulation of these three genes by RNAi does not compromise either viability, or development. In addition, no defects in mRNA export were observed upon simultaneous knockdown of ALY-1, ALY-2 and ALY-3, suggesting that these proteins mediate alternative processes in the nematode, or that their role in transcript export is redundant with other genes (Longman *et al*, 2003). This is in agreement with reports showing that in *Drosophila* ALY proteins are not required for general mRNA transport (Gatfield & Izaurralde, 2002). In contrast, ALY-1 and ALY-2 have been implicated in nuclear retention of a specific mRNA. In *C. elegans*, sex is initially specified by the ratio of X chromosomes to autosomal chromosomes, *i.e.* XX animals are hermaphrodites and XO are males. Female fate requires the genes *tra-1* and *tra-2*, while male cell fate requires inhibition of *tra-2* activity. Kuersten et al.

showed that binding of NXF-2 and ALY-1/2 inhibits nuclear export of *tra-2*. In support of these findings, RNAi against ALY proteins led to 5-7% female occurrence in the progeny (Kuersten *et al*, 2004). It remains to be determined whether m⁵C acts as an intermediate in this process, for example by enhancing binding affinity of ALY proteins to *tra-2* mRNA. Notably, our previous report demonstrated that m⁵C is absent or occurs very rarely in the coding transcripts in *C. elegans*, suggesting that ALY-1/2 could be recognising m⁵C in other molecules (Navarro *et al*, 2020). In addition, the existence of multiple orthologs of ALY genes in *C. elegans* raises the possibility that these proteins could act redundantly or bind differential sets of modified residues in RNA.

Our study presents the first comprehensive investigation of putative readers of m⁵C and its derivatives in a whole organism. This dataset represents an important resource for the discovery of new functions of the RNA m⁵C pathway.

MATERIALS AND METHODS

Genetics

C. elegans strains were grown and maintained as described in Brenner (Brenner, 1974). The strains were kept at 20°C, unless otherwise indicated. HB101 strain *Escherichia coli* was used as food source (*Caenorhabditis* Genetics Center, University of Minnesota, Twin Cities, MN, USA). Bristol N2 was used as the wild type strain.

Oligonucleotide synthesis

The synthesis of 2'-O-methyl-5-hydroxymethylcytidine (hm⁵Cm), 5-hydroxymethylcytidine (hm⁵C) and 5-formylcytidine (f⁵C) phosphoramidite monomers was performed as described in Tanpure & Balasubramanian, 2017.

RNA oligonucleotides (5'-GCXUCCGAUGXUACGGAGGCUGAXC-biotin-3', where X = C, m⁵C, hm⁵C, f⁵C or hm⁵Cm) were synthesised in collaboration with ATDBio, Southampton, UK. Purity and integrity of all modified RNA oligonucleotides were confirmed by LC-MS analysis.

Protein extraction

Nematodes were grown until gravid adults in 140 mm NGM plates seeded with concentrated HB101 *E. coli* and then harvested and washed twice in M9 buffer. A final wash was performed in lysis buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1.5 mM MgCl₂, 0.1% IGEPAL, 0.5 mM DTT) and the animals were resuspended in 4 ml of lysis buffer before the addition of a protease inhibitor cocktail (SIGMAFAST protease inhibitor tablets, Sigma). The animals were pelleted and as much liquid as possible was removed before the samples were frozen as droplets in liquid nitrogen, using a Pasteur pipette. Frozen droplets were transferred to metallic capsules and cryogenically grinded for 25 sec in a mixer (Retsch MM 400 Mixer Mill). The resulting frozen powder was stored at -80°C until the moment of use. The powder was defrosted at 4°C and the lysate was sonicated for 10 cycles of 20 sec, with breaks of 20 sec in between, in a Bioruptor Pico (Diagenode). The sample was centrifuged at maximum speed for 15 min at 4°C and the protein concentration of the supernatant was determined using a BioRad Bradford protein assay (DTT tolerant).

RNA pulldown

RNA pulldown protocol was adapted from Dominissini *et al*, 2012. Magnetic beads (Dynabeads MyOne Streptavidin C1, Invitrogen) were washed twice in 100 mM Tris-HCl pH 7.5, 10 mM EDTA, 1 M NaCl, and 0.1% Tween-20, followed by two washes in lysis buffer. In order to reduce non-specific protein binding to the RNA bait matrix, lysates were pre-cleared by incubation with washed beads for 1 h at 4°C with constant rotation prior to the pulldown. Finally, the following pulldown mix was prepared: 2 mg of protein lysate, 50 U of RNase inhibitor Suprase-IN (Invitrogen), 500 pmol of 25-mer biotinylated RNA bait, up to a final volume of 250 µl in lysis buffer. The mixture was incubated with constant rotation for 2 h at 4°C and then added to 20 µl of streptavidin-conjugated beads for pulldown for another 2 h at 4°C. The beads were washed 3-5 times in 1 ml of lysis buffer and the bound proteins were eluted for mass spectrometry in 50 µl 4% SDS 100 mM TEAB at 95°C for 10 min.

Sample preparation for protein mass spectrometry

Samples were processed and analysed as described in Bensaddek *et al*, 2016. Samples were reduced using 25 mM tris-carboxyethylphosphine TCEP for 30 min at room temperature, then alkylated in the dark for 30 min using 50 mM iodoacetamide. Protein concentration was quantified using the EZQ assay (Thermo Fisher). The lysates were diluted with 100 mM triethyl ammonium bicarbonate (TEAB) 4-fold for the first digestion with endoprotease Lys-C (Fujifilm Wako Chemicals), then further diluted 2.5-fold before a second digestion with trypsin. Lys-C and trypsin were used at an enzyme to substrate ratio of 1:50 (w/w). The digestions were carried out overnight at 37°C, then the tryptic digestion was terminated by acidification with trifluoroacetic acid (TFA) to a final concentration of 1% (v:v). Peptides were desalted using C18 Sep-Pak cartridges (Waters) following manufacturer's instructions, dried and dissolved in 5% formic acid (FA).

Reverse-phase liquid chromatography-MS

RP-LC was performed using a Dionex RSLC nano HPLC (Thermo Fisher Scientific). Peptides were injected onto a 75 μ m x 2 cm PepMap-C18 pre-column and resolved on a 75 μ m x 50 cm RP- C18 EASY-Spray temperature controlled integrated column-emitter (Thermo Fisher Scientific). The mobile phases were: 2% ACN incorporating 0.1% FA (Solvent A) and 80% ACN incorporating 0.1% FA (Solvent B). A gradient from 5% to 35% solvent B was used over 4 h with a constant flow of 200 nL/min. The spray was initiated by applying 2.5 kV to the EASY-Spray emitter and the data were acquired on a Q-Exactive Orbitrap (Thermo Fisher Scientific) under the control of Xcalibur software in a data dependent mode selecting the 15 most intense ions for HCD-MS/MS. Raw MS data was processed using MaxQuant (version 1.3.0.5) (Cox & Mann, 2008).

Mass Spectrometry Data Analysis

Putative readers were ranked based on the median of the intensity-based absolute quantification (iBAQ) values (Schwanhäusser *et al*, 2011). Briefly, for normalisation, iBAQ values were divided by the total sum of intensity of each sample and the median of normalised biological replicates was calculated. A value of 1×10^{-9} (a number 100 times smaller than the smallest value in the dataset) was then added to all median values to calculate fold changes.

SDS-PAGE

Samples were prepared and validation pulldowns were performed as described in “Protein extraction” and “RNA pulldown”, respectively. Pulldown eluates and input controls were

loaded in a 4-12% Bis-Tris gel and ran in NuPAGE MOPS SDS running buffer (Invitrogen) at 200V for 50 min in a XCell SureLock electrophoresis system (Thermo Fisher Scientific) connected to a Bio-Rad PowerPac (Bio-Rad).

Western Blotting

Proteins resolved in SDS-PAGE were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham) using NuPAGE Transfer Buffer (Invitrogen), for 2 h, at 250 mA, 4°C in a Mini Trans-Blot Cell (Bio-Rad). Membranes were blocked in 3% non-fat dry milk in TBS-T buffer for 30 min at room temperature. Membranes were incubated with primary antibody (mouse anti-FLAG M2, Sigma F1804) at 1:1000 dilutions in 3% milk-TBS-T overnight rotating at 4°C. Following three washes in TBST-T for 10 min each, membranes were incubated with secondary antibody diluted in milk/TBS-T for 1–2 hours at room temperature. Following 3 washes in TBS-T, bands were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to manufacturer's instructions. Medical X-ray films (Super Rx, Fuji) were exposed to luminescent membrane for the time required and the films were developed on a Compact X4 automatic X-ray film processor (Xograph Imaging Systems Ltd).

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COMPETING INTERESTS

E.A.M. is a co-founder and director of Storm Therapeutics, Cambridge, UK.

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FIGURE LEGENDS

Figure 1. Identification of RNA modification candidate readers. (a) Molecular baits were designed bearing modifications in three structural contexts: single-strand, double-strand, and loop. m⁵C, hm⁵C, hm⁵Cm and f⁵C monomers were synthesised and incorporated into oligonucleotides tethered to TEG-biotin. Blue circles indicate modified positions. Pre-cleared protein lysate from synchronised adult populations was incubated with unmodified or modified biotinylated RNA baits. Streptavidin-conjugated magnetic beads were used to capture proteins that interact with the RNA. Following washes and elution, proteins were processed and identified by RP LC-MS. (b) Scatter plot of proteins bound to modified versus unmodified RNA baits. Median of the normalised iBAQ values were plotted. Proteins uniquely enriched in m⁵C, hm⁵C, hm⁵Cm and f⁵C pulldowns are highlighted in mustard. Proteins were considered candidate readers whenever the fold-change of iBAQ values over the “beads only” and “unmodified C” controls was >2.0 and >1.5, respectively. n = 3 independent biological replicates.

Figure 2. Phenotype enrichment for proteins identified as unique candidate readers. (a) Venn diagram showing the number of enriched proteins detected by mass spectrometry in pulldowns using RNA with different m⁵C, hm⁵C, hm⁵Cm or f⁵C. (b) Phenotype enrichment analysis of unique binders of m⁵C, hm⁵C, hm⁵Cm or f⁵C. Enrichment analysis was performed with the Wormbase Enrichment Suite. Colour code: m⁵C in orange, hm⁵C in purple, hm⁵Cm in

red and f⁵C in blue.

Figure 3. ALY-1 and ALY-2 preferentially bind to m⁵C-modified RNA *in vitro*. (a) Protein sequence alignment of ALYREF and ALY-1/ALY-2 using Clustal Omega. Residues in ALYREF required for m⁵C-binding previously identified by Yang *et al.* are highlighted in red box. “*” represents identical amino acids while “:” and “.” represent amino acids with strongly and weakly similar properties, respectively. (b) Anti-FLAG western blotting (left) showing enrichment of ALY-1 and ALY-2 following pulldown using m⁵C-modified RNA oligos and relative quantification (right). Mean ± SD, quantification performed using ImageJ, n = 2 independent biological replicates. (c) Overlay of ALY-1 (yellow) or ALY-2 (pink) with ALYREF (grey) structures. ALY-1 and -2 structures were predicted using the SWISS-Model platform which were aligned against a ALYREF crystal structure (PDB: 3ULH). The overall RRM folds are conserved in ALY-1 and -2. (d) Sequence alignment of RRM in m⁵C unique binders with ALYREF using Muscle. Black squares highlight consensus sequences RNP1 and RNP2 that are found in RRM. “*” represents identical amino acids while “:” and “.” represent amino acids with strongly and weakly similar properties, respectively. Amino acids highlighted in pink are identical to the reference sequence (ALYREF).

FIGURE 1

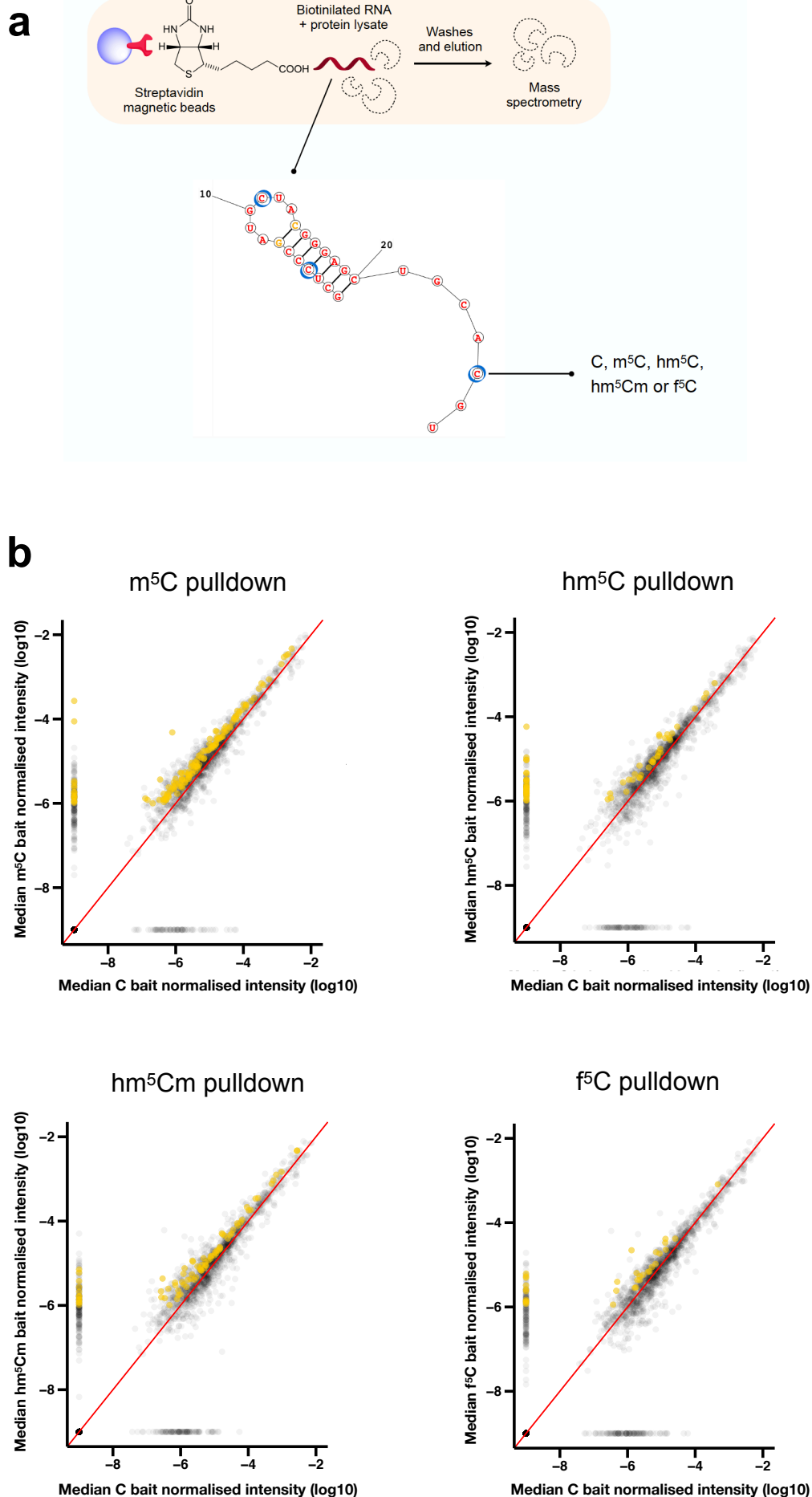
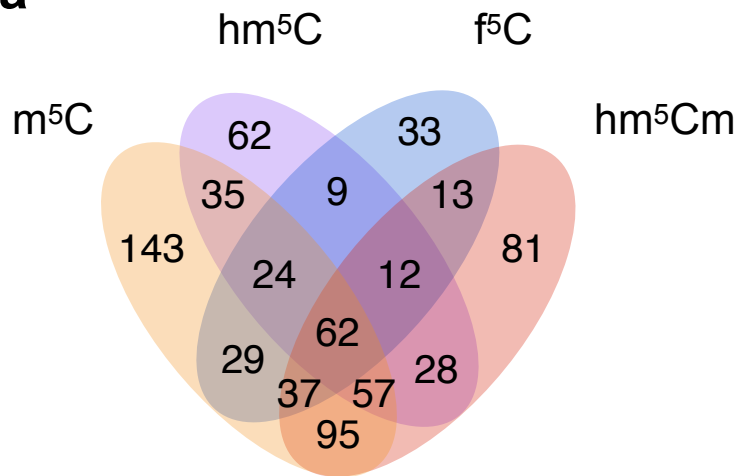


FIGURE 2

a



b

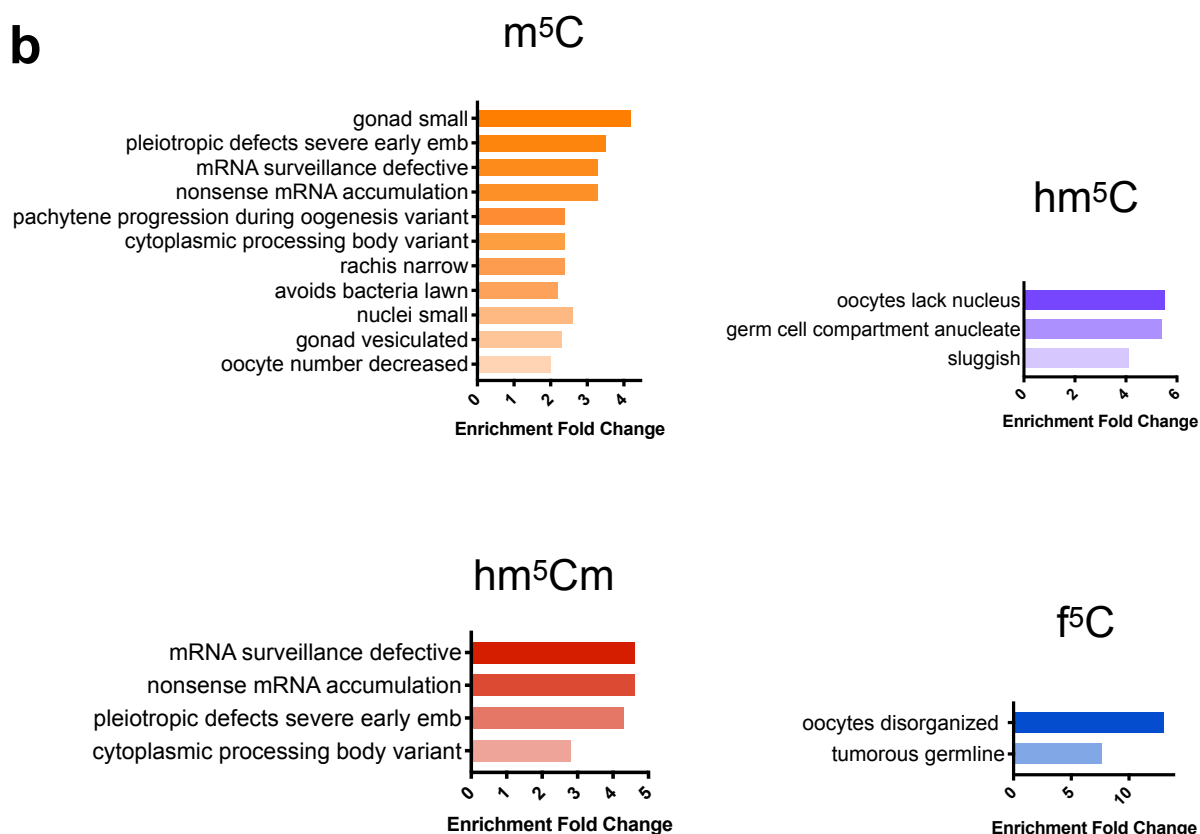


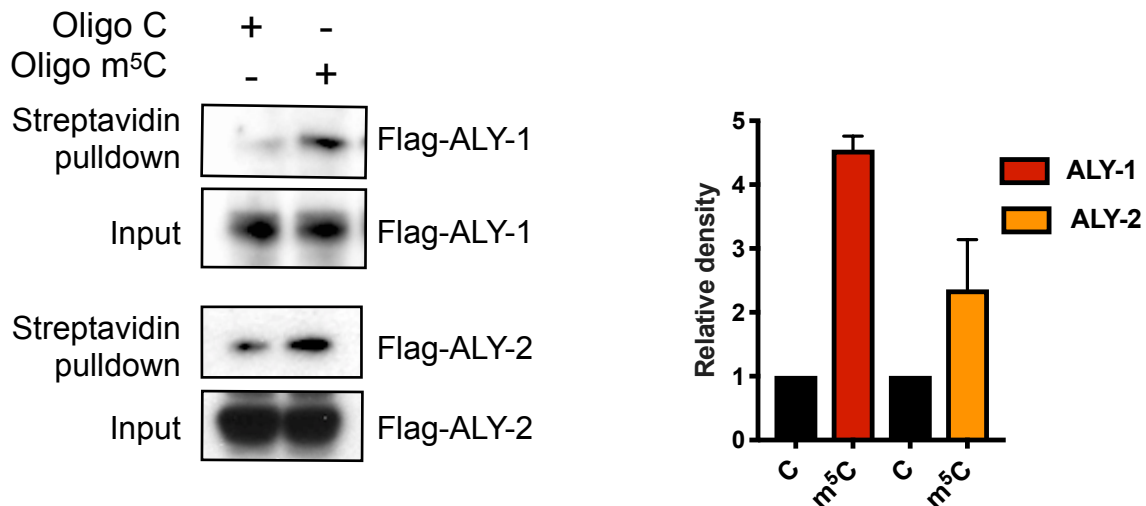
FIGURE 3

a

ALYREF VSDADIQELFAEFGLTKKAAVHYDRSGRSLGTADVHFERKADALKAMKQYNGVPLDGRPM
 ALY-1 VHSGLRQLFAEFKI-RNVSVNFNEHGKPVGTGDVTLPKRH-ADRLIQKFAGVSLDGKEI
 * ..*:::***** :::*::: * : **.* : : * : :::: ** ****: :

ALYREF VSDADIQELFAEFGLTKKAAVHYDRSGRSLGTADVHFERKADALKAMKQYNGVP
 ALY-2 VISSDLQELFGAFNL-HKVSVMFNENGGAAGTGDITLK-KYDADRLIQKFAGVA
 * .*:*****. *. :*:*****. * : **.* : : * ** : :::: **

b



c

ALY-1 vs. ALYREF

ALY-2 vs. ALYREF

Overlay



e

