Limits in the detection of m^{6}A changes using MeRIP/m^{6}A-seq

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Abstract

Recent studies have revealed that many cellular mRNAs contain the modified base m^{6}A and have suggested that various stimuli can lead to changes in m^{6}A. The most common method to map m^{6}A and to predict changes in m^{6}A between conditions is methylated RNA immunoprecipitation sequencing (MeRIP-seq), through which methylated regions are detected as peaks in transcript coverage from immunoprecipitated RNA relative to input RNA. Here, we generated replicate controls and reanalyzed published MeRIP-seq data to estimate reproducibility across experiments. We found that m^{6}A peak overlap in mRNAs varies from ~30 to 60% between studies, even in the same cell type. We then assessed statistical methods to detect changes in m^{6}A peaks as distinct from changes in gene expression. However, we detected few changes under most conditions and were unable to detect consistent changes across studies using similar stimuli. Overall, our work identifies limits to MeRIP-seq reproducibility in the detection both of peaks and of peak changes and proposes improved approaches for analysis of peak changes.

Introduction

Methylation at the *N*6 position in adenosine (m^6A) is the most common internal modification in eukaryotic mRNA. A methyltransferase complex composed of METTL3, METTL14, WTAP, VIRMA, and other cofactors catalyzes methylation at DRACH/DRAC motifs, primarily in the last exon (1,2). Most m^6A methylation occurs during transcription (3). The modification then affects mRNA metabolism through recognition by RNA-binding proteins that regulate processes including translation and mRNA degradation (4–9). However, whether m^6A is lost and gained in response to various physiological changes remains contentious (3,10–15). To assess the evidence for proposed dynamic changes in m^6A, a reliable and reproducible method to detect changes in methylation as distinct from changes in gene expression is necessary.

The first and most widely-used method to enable transcriptome-wide studies of m^6A, MeRIP-seq or m^6A-seq, involves the immunoprecipitation of m^6A-modified RNA fragments followed by peak detection through comparison to background gene coverage (16,17). A second method has since been developed, miCLIP or m^6A-CLIP, which involves crosslinking at the site of antibody binding to induce mutations during reverse transcription for single-nucleotide detection of methylated bases (2,18). MeRIP-seq is still more often used than miCLIP, despite less precise localization of m^6A to peak regions of approximately 50-200 base pairs that can contain multiple DRAC motifs, since it follows a simpler protocol, requires less starting material, and generally produces higher coverage of more transcripts. Antibodies for m^6A can also detect a second base modification, N^6,2′-*O*-dimethyladenosine (m^6A_m), found at a lower abundance than m^6A and located at the 5′ ends of select transcripts (15,18). We thus refer to the base modifications detected through MeRIP-seq collectively as $m^6A_{(m)}$, although most are likely m^6A. As of late 2018, over fifty studies have used MeRIP-seq to detect $m^6A_{(m)}$ in mammalian mRNA (**Supplementary Table 1**).

Although MeRIP-seq can reveal approximate sites of $m^6A_{(m)}$, it cannot be used to quantitatively measure the fraction of transcript copies that are methylated (19). Therefore, studies of m^6A variation in response to stimuli estimate differences at individual loci through changes in peak presence or peak height. Using these approaches, studies have reported changes to m^6A with heat shock, microRNA expression, transcription factor expression, cancer, oxidative stress, human immunodeficiency virus (HIV) infection, Kaposi's sarcoma herpesvirus (KSHV) infection, and Zika virus infection, including hundreds to thousands of changes in enrichment at specific sites (20–29). Statistical approaches to analysis have only recently been published and there have been no comprehensive evaluations of methods to detect changes in m^6A based on MeRIP-seq data (30,31). Thus, while these studies could suggest that m^6A is highly variable in response to diverse stimuli, they have applied inconsistent analysis methods to detect changes in m^6A and often don't control for differences in RNA expression or typical variability in peak heights between replicates. In some cases, these studies have reported m^6A changes based on simple differences in peak count (24,26,27,32). However, others have applied statistical tests or thresholds for differences in immunoprecipitated (IP) over input fraction enrichment and visual analysis of coverage

plots, and have reported fewer m^6A changes or suggested that m^6A is a relatively stable mark (33,34). Since there is noise in MeRIP-seq, multiple replicates are necessary to estimate variance and statistically identify the effects of experimental intervention, as in RNA-seq (35–37). However, only one MeRIP-seq study to date has used more than three replicates per condition (34), while ten have used only one (17,20,32,33,38–43), suggesting that most studies may not have enough power to detect changes in $m^6A_{(m)}$.

To re-evaluate the evidence for $m^6A_{(m)}$ changes under various conditions, we first examined the variability in $m^6A_{(m)}$ detection across replicates, cell lines, and experiments using our own negative controls (12 replicates) as well as 24 published MeRIP-seq data sets. We then defined appropriate statistical methods to detect differences in IP enrichment using biological negative and positive controls for m^6A changes. We found that these methods are limited by noise, including biological variability from changes in RNA expression and technical variability from immunoprecipitation and sequencing that limits reproducibility across studies. Our results suggest that the scale of statistically detectable $m^6A_{(m)}$ changes in response to various stimuli is orders of magnitude lower than the scale of changes reported in many studies. However, we also found that the majority of sites could be missed when using only 2-3 replicates. We use our results to propose approaches to MeRIP-seq experimental design and analysis to improve reproducibility and more accurately measure differential regulation of $m^6A_{(m)}$ in response to stimuli. These data emphasize the need for further research and alternative assays, for example recently developed endoribonuclease-based methods (44,45) or direct RNA nanopore sequencing (46), to resolve the extent to which m^6A changes in response to specific conditions.

Results

Detection of peaks across replicates, experiments, and cell types

The first steps in MeRIP-seq data analysis are to align sequencing reads to the genome or transcriptome of origin and to identify peaks in transcript coverage in the IP fraction relative to the input control. Several methods have been developed for MeRIP-seq peak detection, including exomePeak, MeTPeak, MeTDiff, and bespoke scripts. Another method often used for MeRIP-seq peak detection is MACS2, which was originally designed to detect protein binding sites in DNA from chromatin immunoprecipitation sequencing (ChIP-seq). We compared $m^6A_{(m)}$ peak detection by exomePeak, MeTPeak, MeTDiff, and MACS2 (31,47–49) in seven replicates of MeRIP-seq data obtained from mouse cortices under basal conditions (34), and in 12 replicates of MeRIP-seq data we generated from human liver Huh7 cells. The intersect between all tools tested was high and we saw minimal differences in DRAC motif enrichment (**Supplementary Figure 1a**). Since MACS2 is the most commonly used tool and was previously used to compare MeRIP-seq experimental methods (42), we used MACS2 for the remainder of our analyses.

We next defined the threshold of detection for peaks in MeRIP-seq data in terms of coverage. For $m^6A_{(m)}$ peak detection, a transcript must be sufficiently expressed for enrichment by $m^6A_{(m)}$ antibody and adequate sequencing coverage in both the IP and input fractions. Because previous reports have suggested that $m^6A_{(m)}$ presence does not decrease with expression level (9), we assume that the detection of fewer peaks as read counts at a gene or peak decrease indicates inadequate coverage. To estimate the level of coverage necessary for peak detection, we analyzed the percent of genes with at least one, two, or three peaks relative to mean input transcript coverage in both the mouse cortex and Huh7 cell data (**Figure 1a**). Based on the upper shoulders of the sigmoidal curves as the percent of genes with peaks begins to plateau, we estimate that gene coverage of approximately 10-50X is necessary to avoid missing peaks based on insufficient coverage. Input RNA-seq coverage of peak regions alone supports a similar threshold; few peaks are detected with median input read counts below 10 across replicates (**Supplementary Figure 1b**).

To evaluate the reproducibility of MeRIP-seq data, we next examined the consistency of $m^6A_{(m)}$ peak calling between replicates. Previous studies have reported that peak overlap between replicates is approximately 80% (9,16,50,51). Similarly, we found that between two replicates, $\log_2$ enrichment of IP over input reads at detected peaks showed a Pearson correlation of approximately 0.8 to 0.86 (**Supplementary Figure 1c**, top). A single replicate captured a median of 78% of the peaks found in seven replicates of mouse cortex data and 67% of peaks found in twelve replicates of Huh7 cell data. The number of detected peaks increased log-linearly with the addition of more replicates, such that with three replicates, 84-92% of the peaks found with 7-12 replicates were detected (**Figure 1b**, top). Conversely, the number of peaks in common across replicates decreased as the number of replicates increased, such that while ~80% of peaks were detected in at least two replicates, only ~60% were detected in six replicates for both data sets and ~30% in all twelve replicates of Huh7 cell data (**Figure 1b**, bottom). Detection of peaks in more replicates did not increase DRAC motif enrichment (**Supplementary Figure 1c**, bottom). These results suggest that many $m^6A_{(m)}$ sites may be missed in studies that use one to three replicates, and that increasing replicates could enable detection of more peaks. However, not all peaks correspond to true $m^6A_{(m)}$ sites. A recent reanalysis found that at least one published data set lacked enrichment for the canonical DRAC motif under MeRIP-seq peaks, suggesting a high ratio of false to true positives (3).

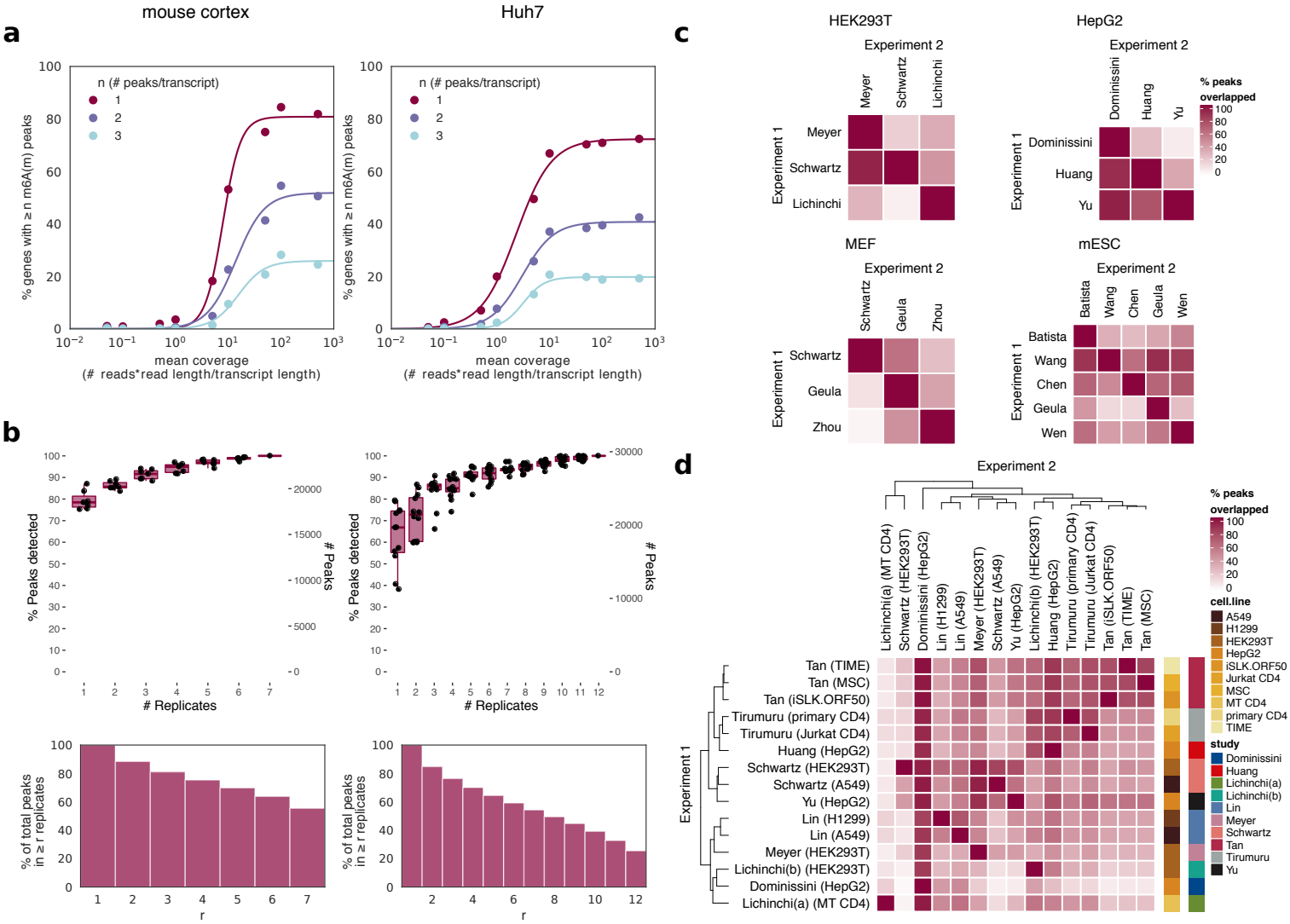

Figure 1: Thresholds and reproducibility of peak detection. a) $m^6A_{(m)}$ site detection in MeRIP-seq data from mouse cortex (left) and human liver cells (Huh7, right) shows saturation of peak detection as transcript coverage approaches 10-50X for replicates at basal conditions, with peaks merged from all replicates. **b)** The total number of peaks captured increases with more replicates, with single replicates capturing a median of 67-79% of total peaks depending on study. Boxes span the 1st to 3rd quartiles of distributions for random subsamples of replicates, with lines indicating the median number of peaks, and whiskers showing the minimum and maximum points within ±1.5x the interquartile distance from the boxes. Jittered points show results for each random subsample (a total of 6 subsamples per replicate number for the mouse cortex data and 12 for the Huh7 data). **c)** Peak detection between studies that used the same cell type shows variable overlap. Overlap was calculated as the percent of peaks detected in Experiment 1 with an overlap of ≥ 1 base pair with peaks from Experiment 2. **d)** Peak detection across tissue and cell types shows samples from the same study cluster better together than samples from the same tissue. Studies used in (c) and (d) are described in **Supplementary Table 2**.

The number of peaks detected across studies varies. Given that coverage affects peak detection, we hypothesized that variation in sequencing depth could contribute to differences in peak count. Zeng et al. (2018) reported that peak count begins to saturate by around 20 million reads by subsampling data

141 within individual studies(42). However, we found that there is no positive correlation between peak count
142 and input or IP sequencing depth across data sets from different published studies, each of which had 3-
143 81M reads per replicate (input Pearson's R = -0.37, p = 0.015; IP Pearson's R = -0.18, p = 0.24)
144 (**Supplementary Table 2, Supplementary Figure 1d**). This implies that other experimental factors
145 contribute to the variability of peak counts across studies.
146 We next analyzed the overlap of peaks among studies and found similar inconsistency in peak
147 localization on transcripts. Within four commonly used cell types, the percent of peaks detected in one
148 experiment that were also detected in a second varied among pairs of studies from as low as 2% of peaks
149 to as high as 90%, filtering for transcripts expressed above a mean of 10X input coverage in both (**Figure**
150 **1c,** there were insufficient shared transcripts at 50X for most combinations to use that threshold). In fact,
151 peaks showed higher overlap within different cell types from the same study than within the same cell
152 type from different studies, suggesting that MeRIP-seq data is prone to strong batch effects (**Figure 1d**).
153 We were unable to identify a link between peaks called and differences among experimental protocols
154 used (summarized in **Supplementary Table 2**). Overall, most percent overlaps of $m^6A_{(m)}$ peaks fell
155 between ~30% (1st quartile) and ~60% (3rd quartile). These results thus suggest that multiple labs running
156 MeRIP-seq on the same cell type will not detect the same $m^6A_{(m)}$ sites.
157
158 *Detection of changes in peaks between conditions*
159 Following $m^6A_{(m)}$ peak detection, many studies seek to compare the expression of peaks between
160 two conditions. Looking at plots of IP and input gene coverage under different conditions can help
161 evaluate the evidence for peak changes (33), however, statistical or heuristic methods are necessary to
162 narrow down a list of candidate sites to plot. Several tools used for statistical analysis by the studies in
163 **Supplementary Table 1** or for other types of RNA IP sequencing assays model peak counts using either
164 (a) the Poisson distribution, in which the variance of a measure (here, read counts) is assumed to be
165 equal to the mean (MeTDiff), or (b) the negative binomial distribution, in which a second parameter allows
166 for independent adjustment of mean and variance (QNB and two implementations of a generalized linear
167 model approach using DESeq2 or edgeR, **Table 1**) (30,31,52–54). In the mouse cortex and Huh7 cell
168 data, we found that, similar to RNA-seq data (24,53,55), the variance in read counts under peaks
169 exceeded their mean, indicative of overdispersion (**Supplementary Figure 2a**). The log likelihood (the
170 probability of an observation given a distribution with known parameters) for our sample also fell within
171 the distribution of expected log likelihoods for the negative binomial distribution (bottom) but not the
172 Poisson distribution (top) (**Figure 2a**). Thus, the negative binomial distribution captures the mean-
173 variance relationship in MeRIP-seq data, suggesting that tools that account for overdispersion better
174 model the distribution of read counts at $m^6A_{(m)}$ peaks than tools that do not.
175
176

177 **Table 1:** Statistical methods for the detection of peak changes

Method	Read count distribution	Publication
MeTDiff	Poisson	Cui et al. (2018)
Quad-negative binomial (QNB)	Negative binomial	Liu et al. (2017)
GLM (DESeq2)	Negative binomial	based on Park et al. (2014) method for HITS-CLIP
GLM (edgeR)	Negative binomial	

178

179 We next defined positive and negative controls to evaluate tool performance for detection of
180 changes in $m^6A_{(m)}$ peaks. Past evaluations of methods have used data sets in which methylation
181 machinery genes or the methyl donor were disrupted compared to baseline conditions as positive
182 controls, and have simulated negative controls by randomly swapping labels in the positive controls
183 (30,31). However, swapping labels for conditions that may feature differences in gene expression in
184 addition to m^6A levels could unrealistically increase variance in read counts within groups. Therefore, we
185 instead used the two data sets from mouse cortex and Huh7 cells, which each comprised many replicates
186 at baseline conditions (n=7 and n=12, respectively), as negative controls. We randomly divided the
187 mouse cortex data into two groups of three replicates for comparison and divided the Huh7 replicates by
188 lab of incubation, which did not affect sample clustering (**Supplementary Figure 2b**). We would expect to
189 see minimal changes in IP enrichment at m^6A peaks between groups for our negative controls, whereas
190 our positive controls, which featured genetic or chemical interference with the m^6A machinery, should
191 show discernible differences in peaks (summarized in **Supplementary Table 3**).
192 When we compared the negative and positive controls, we found that the percent of peak
193 changes called below a p-value threshold of 0.05 were similar (**Figure 2b**). With all tools except MeTDiff,
194 a knockout of Mettl3 showed the largest effects on m^6A (56), while fewer significant peaks in other
195 positive controls suggested variable effects of the positive control conditions on $m^6A_{(m)}$, possibly related to
196 knockdown or overexpression efficiency (7,33,57–61). In the absence of true differences between groups,
197 p-value distributions should be uniform for well-calibrated statistical tests, meaning that ~5% of peaks
198 should have p-values < 0.05 for the negative controls. MeTDiff reported an excess number of sites with p-
199 values below 0.05 (**Supplementary Figure 2c**) and identified a higher percentage of sites as differentially
200 methylated in the mouse cortex negative control data set than in all but two positive controls (**Figure 2b**).
201 By contrast, the generalized linear models (GLMs) and QNB showed uniform to conservatively shifted p-
202 value distributions, with differences between the mouse cortex and Huh7 data sets (**Supplementary**
203 **Figure 2c**), suggesting fewer false positives than MeTDiff.
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205
206

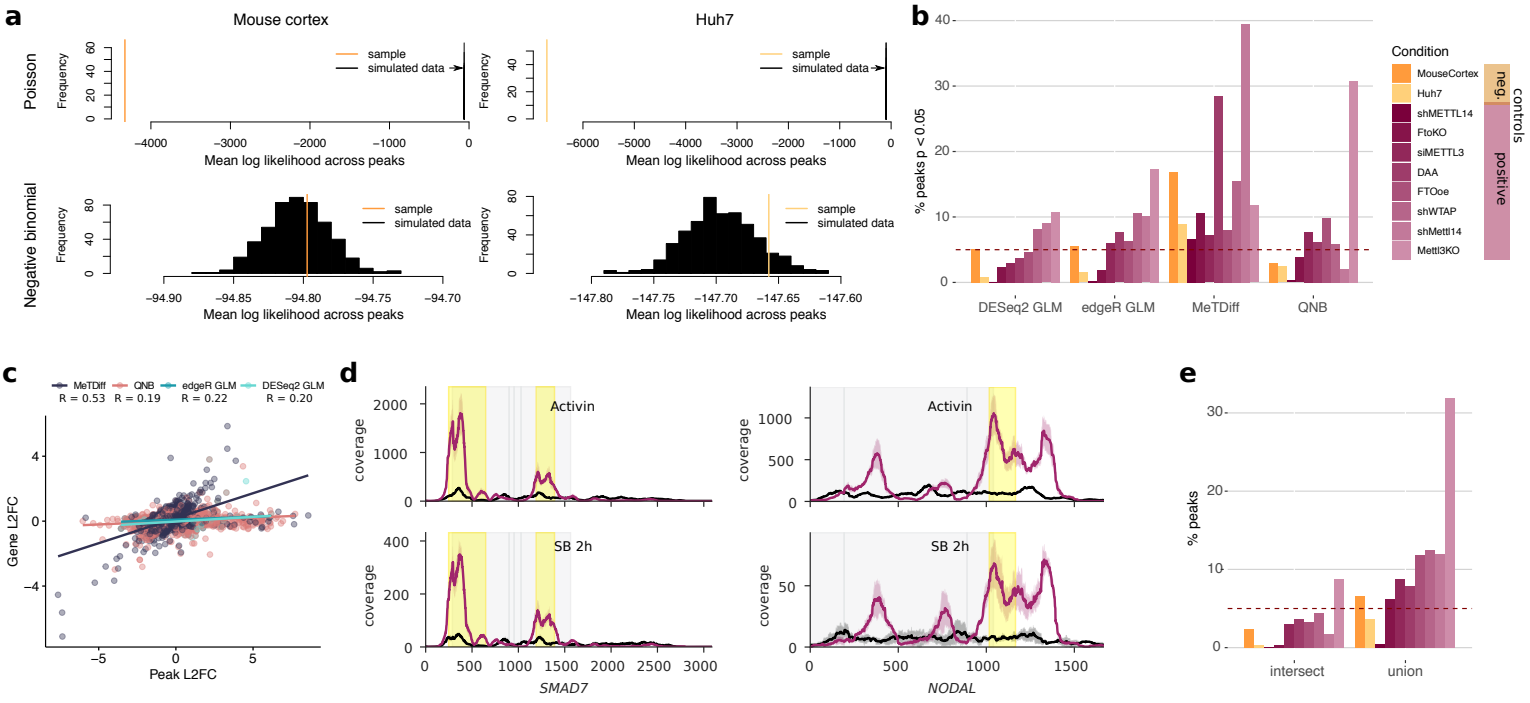

Figure 2: Analysis of methods to detect peak changes disproportional to gene expression changes. a) A comparison of Poisson (above) and negative binomial (below) models for read counts under peaks. The negative binomial mean log likelihood of the sample data fell within the 63rd and 91st percentiles of 500 simulations for mouse cortex and Huh7 cell data, respectively, while the Poisson model failed to capture the sample distributions. **b)** The percent of sites below an unadjusted p-value threshold of 0.05 for different methods (described in **Table 1**) to detect differential methylation in negative controls between two groups at baseline conditions and positive controls in which methylation processes were disrupted with respect to baseline conditions (**Supplementary Table 3**). The line at 5% indicates the expected proportion of sites given a uniform p-value distribution (see **Supplementary Figure 2c**), while colours indicate negative (orange) and positive (purple) control experiments. **c)** The correlation between change in gene expression and change in peak expression between conditions for sites identified as differentially methylated in the eight positive control experiments. Pearson's R = 0.22, 0.20, 0.53, and 0.19 for edgeR, DESeq2, MeTDiff, and QNB, respectively, with p = 7.0E-4, 0.10, 1.1E-71, and 2.9E-21. **d)** Coverage plots showing changes in peak expression are proportional to changes in gene expression for genes identified using MeTDiff as less methylated after two hours of treatment using an activin-NODAL inhibitor, SB431542 (SB), than with incubation in the presence of activin by Bertero et al. (2018). Lines show the mean coverage across three replicates, while shading shows the standard deviation. Peaks detected as significantly changed are highlighted in yellow. Coding sequences are shown in grey. **e)** The intersect and union of peaks with $p < 0.05$ from DESeq2, edgeR, and QNB from (b), coloured as in (b).

To ensure significant peaks detected by each of the tools reflected changes in IP enrichment independent of differential gene expression, we measured the correlation between changes in IP read counts at peak sites and changes in input read counts across their encompassing genes. For significant peaks (FDR-adjusted p-value < 0.05) from the positive controls, correlation between $\log_2$ fold change in

211 peak IP and gene input read counts was low for the GLMs and QNB (Pearson's R = 0.18 to 0.21, 2.9E-21
212 < p < 0.1) but reached 0.53 (p = 2.3E-74) for MeTDiff (**Figure 2c**). The higher correlation for MeTDiff was
213 driven by peaks with proportional changes in IP and input levels, which suggests that MeTDiff often
214 detects differential expression of methylated genes rather than differential methylation and is therefore of
215 relevance for published studies that have used MeTDiff (22,62). Plotting coverage for genes reported as
216 differentially methylated in one of these studies with the y-axis scaled separately per condition confirmed
217 that changes in m^6A identified by MeTDiff were proportional to changes in gene expression (**Figure 2d**)
218 (22). Given these results, QNB or the GLM implementations are better methods than MeTDiff to detect
219 differential methylation. Taking the intersect of significant peaks for the GLMs and QNB may help
220 determine the most probable sites, while taking the union of predictions provides a less conservative
221 approach to selecting sites for further validation (**Figure 2e**). However, there were still significant peaks
222 for which the difference between peak $\log_2$ fold change and gene $\log_2$ fold change was close to zero,
223 particularly with QNB (**Supplementary Figure 2d**). For the remainder of our analyses, we therefore
224 added a filter to the combined predictions from QNB and the two GLMs for difference in peak and gene
225 $\log_2$ fold change ≥ 1, with an additional filter where noted for peak read counts ≥ 10 across all replicates
226 and conditions to ensure sufficient coverage for peak detection.
227
228 *Reanalyzing peak changes between conditions*
229 We next estimated the scale of statistically detectable peak changes under various conditions
230 using our approaches and compared these results to previously reported estimates of these changes
231 (**Figure 3a**, **Supplementary Table 4**). We identified fewer peaks as differentially methylated than
232 originally reported under most conditions, with zero to hundreds of peaks significantly changed
233 (depending on experiment and method), versus hundreds to over ten thousand described in publications
234 (22–26,34,59,62–66). Notably, knockdown of Zc3h13 did appear to disrupt $m^6A_{(m)}$, suggesting the gene
235 does participate in methylation as recently suggested (64). Another study reported that activin treatment
236 of human pluripotent stem cells led to differential methylation of genes that encode pluripotency factors
237 (22). However, our reanalysis found few peak changes that passed our filters for significance, fold
238 change, and expression (minimum input read count across peaks ≥ 10) and no enrichment for
239 pluripotency factors among affected genes. Without the thresholds for fold change and expression, the
240 adjusted p-value for enrichment of the KEGG pathway "signaling pathways regulating pluripotency of
241 stem cells" dropped to 0.15 based on three genes, LEFTY2, FZD28, and FGFR3 (**Supplementary**
242 **Figure 3a**). Interestingly, the minimum read threshold made a particularly dramatic difference in the case
243 of a recent study that looked at the effects of knocking down the histone methyltransferase SETD2 on
244 m^6A in mRNA, with 2064/2065 sites predicted by QNB falling below that threshold due to low input
245 coverage in the first and second replicates (**Supplementary Figure 3b-e**) (65). We could not compare
246 our approach to results reported by Su et al. (2018), who found 6,024 peaks changed with R2HG

247 treatment, Zeng et al. (2018), who found 465-599 peaks changed between tumour samples, or Ma et al.
248 (2018), who found 12,452 peaks were gained and 11,192 lost between P7 and P20 mouse cerebella, as
249 each relied on a single replicate per condition (40–42).

250 Multiple studies have investigated $m^6A_{(m)}$ in the context of heat shock, HIV infection, KSHV
251 infection, and dsDNA treatment or human cytomegalovirus (HCMV) infection (**Supplementary Table 5**).
252 Since each step in MeRIP-seq analysis risks introducing false negatives, we cannot rule out consistent
253 changes between studies that used similar experimental interventions based on statistical detection
254 alone. Therefore, we plotted coverage for specific genes reported as differentially methylated to evaluate
255 reproducibility across these studies. Zhou, et al. (2015) reported 5′ UTR methylation of *Hspa1a* with heat
256 shock (20). Coverage was too low for untreated controls to determine if *Hspa1a* was newly methylated or
257 newly expressed with heat shock based on our alignment of their data using STAR (67). We were also
258 unable to detect a change in methylation of *HSPA1A* using data from other heat shock studies, including
259 a new data set from a B-cell lymphoma cell line and a published miCLIP data set, although coverage was
260 again low (**Figure 3b**) (4). Lichinchi, et al. (2016) reported that 56 genes showed increased methylation
261 with HIV infection in MT4 T-cells, with enrichment for genes involved in viral gene expression (25).
262 Specific genes, for example *PSIP1*, in which we also detected a peak using MACS2 and see a change in
263 the peak when plotting coverage using the data from Lichinchi et al. (2016), did not show the same
264 changes in data from two other $CD4^+$ cell types, primary $CD4^+$ cells and Jurkat cells (**Figure 3c**) (68). Two
265 other studies both used MeRIP-seq to detect m^6A in *IFNB1* induced through dsDNA treatment or infection
266 by the dsDNA virus HCMV (69,70). The different treatments, time points and use of a fibroblast cell line
267 versus primary foreskin fibroblasts make it difficult to compare $m^6A_{(m)}$ changes between the two
268 experiments. Nevertheless, using QNB and the GLM approaches, we found five peaks in three genes
269 (*AKAP8*, *SUN2*, and *TMEM140*) that showed significant changes both after 12 h of dsDNA treatment
270 compared to untreated controls (69) and after 6 h post-HCMV infection compared to 72 h, when interferon
271 levels have declined (70) (**Figure 3d**). Overall, we were unable to detect the same changes in $m^6A_{(m)}$
272 across studies of heat shock or HIV and few common changes in the response to dsDNA, but cell line-
273 specific differences in $m^6A_{(m)}$ regulation and differences in experimental protocols could account for some
274 of the variability among these studies.

275 While we did not have MeRIP-seq data for two studies from exactly the same conditions and cell
276 lines to compare, two studies both used cell lines derived from iSLK to study the effects of KSHV on host
277 m^6A (27,28). Both suggested that KSHV infection could decrease the number of m^6A sites in host
278 transcripts. Hesser et al. (2018) found that lytic KSHV infection decreased the number of peaks on host
279 transcripts by >25%; Tan et al. (2018) suggested a loss of 17-59% of peaks in two different cell types, but
280 that $m^6A_{(m)}$ peak fold enrichment showed better clustering by cell type than by infection status. Neither
281 discussed specific genes that showed differential methylation with lytic infection. For our comparison of
282 $m^6A_{(m)}$ peak changes in these data sets, we identified probable changes in peaks based on statistical

283 significance using QNB or the GLMs with $\log_2$ fold change difference between peaks and genes of ≥1. We
284 detected 80 peak changes in the data from Hesser et al. (2018) and 18 in the data from Tan et al. (2018)
285 but found no peaks that changed in both iSLK data sets with lytic KSHV infection.

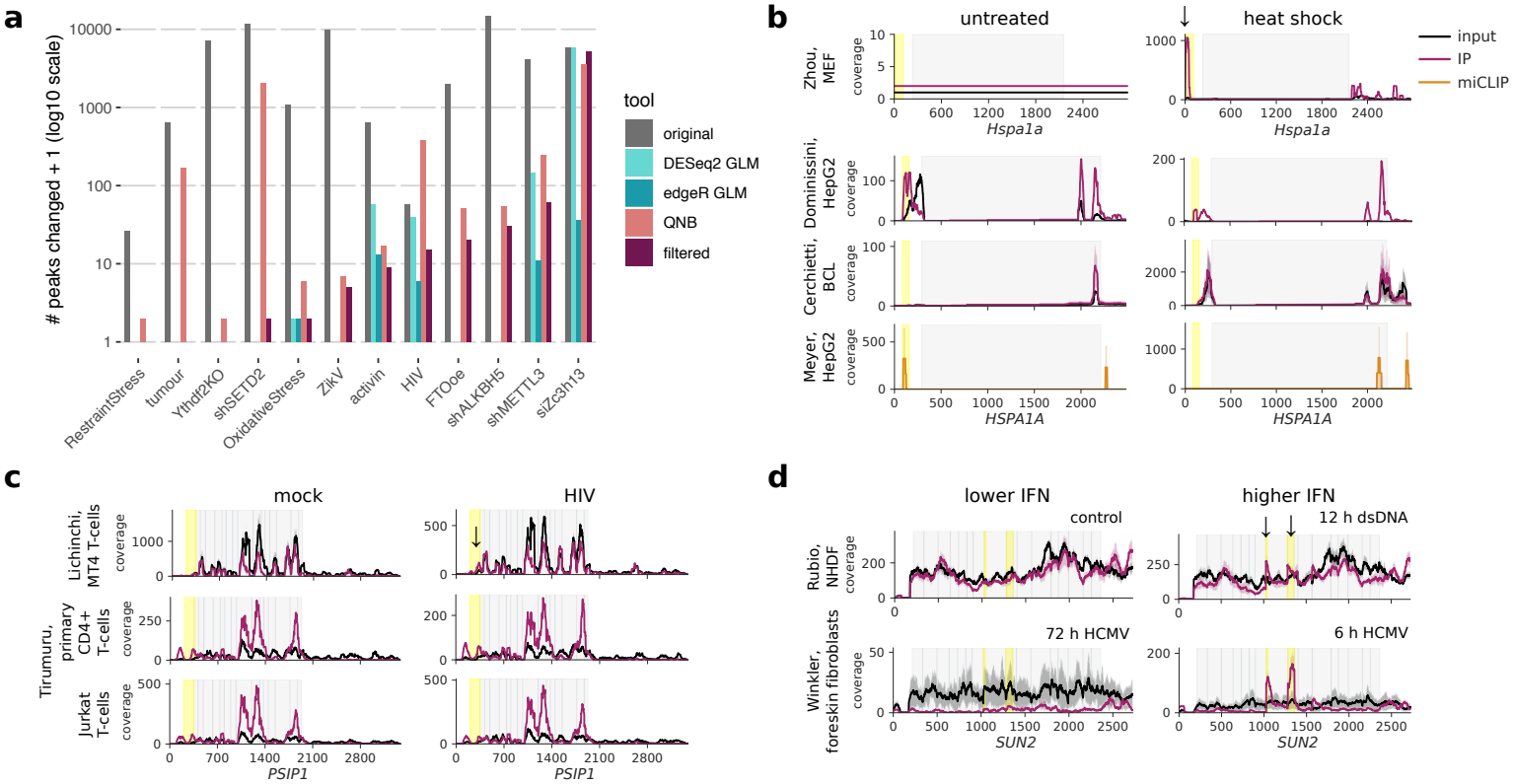

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Figure 3: Changes in peaks between conditions. a) Detected $m^6A_{(m)}$ changes in ten published data sets that measured $m^6A_{(m)}$ peak changes between two conditions **(Supplementary Table 4)**. The number of peaks detected as changed in the original published analyses are compared to the number of peaks with FDR-adjusted p-values < 0.05 in our reanalysis using DESeq2, edgeR, or QNB, and taking the union of results from these three tools with additional filters for $\log_2$ fold difference in peak and gene changes of ≥1 and peak read counts ≥10 across all replicates and conditions ("filtered"). **b)** Gene coverage plots for *Hspa1a* in mouse embryonic fibroblasts (MEFs) and *HSPA1A* in human cells (HepG2 and BCL) before and after heat shock. Input coverage is shown in black and IP coverage in raspberry, with putative m^6A peaks changed highlighted in yellow and marked by arrows. miCLIP coverage for an experiment in HepG2 cells is shown in orange. **c)** Coverage plots for *PSIP1*, which was reported to have a change in 5′ UTR m^6A with HIV infection by Lichinchi et al (2016). **d)** Coverage plots for *SUN2*, in which we detected changes in m^6A with HCMV infection and dsDNA treatment suggesting a possible increase in methylation under higher interferon conditions. Lines in coverage plots (**b-d**) show the mean across all replicates for each experiment, while shading shows the standard deviation. Coding sequences are shown in grey.

287 *MeRIP-RT-qPCR validation*

288 Although statistical approaches revealed fewer changes in $m^6A_{(m)}$ with various stimuli than
289 published estimates, and we were unable to confirm changes in $m^6A_{(m)}$ methylation of specific genes
290 across studies of similar conditions, many of the studies we looked at do include additional validation of
291 $m^6A_{(m)}$ changes from MeRIP-seq using MeRIP-RT-qPCR. Recently it was shown that MeRIP-RT-qPCR
292 can capture differences in m^6A:A ratios at specific sites (34), but it is unknown how MeRIP-RT-qPCR is
293 affected by changes in gene expression. To test this, we ran MeRIP-RT-qPCR on in vitro transcribed RNA
294 oligonucleotides that lacked or contained m^6A spiked into total RNA extracted from Huh7 cells
295 (**Supplementary Table 6**). We found that MeRIP-RT-qPCR was able to detect the direction of change in
296 m^6A levels at different spike-in concentrations (**Figure 4a-b**). However, technical variation could also lead
297 to spuriously significant differences between two dilutions of in vitro controls with the same ratio of m^6A:A.
298 For example, a comparison of m^6A enrichment between 30% methylated spike-ins at 0.1 fmol and 1 fmol
299 returned a p-value of 0.004 (unpaired Student's *t*-test).

300 We next assessed the correlation between m^6A enrichment observed using MeRIP-seq and
301 MeRIP-RT-qPCR using data from our recent work on changes in m^6A in Huh7 cells following infection by
302 different viruses. For those experiments, we again selected peaks that change based on results from
303 QNB and the GLM approaches. We found that the magnitude of changes in common among viruses
304 correlated between MeRIP-seq and MeRIP-RT-qPCR, both across peaks (Pearson's R = 0.57, p = 3.7E-
305 6) and within single peaks across viruses (13 out of 19 peaks showed positive correlations, four of which
306 had p-values < 0.05 with three data points) (**Figure 4c, Supplementary Figure 4**). Given the correlation
307 we found between MeRIP-seq and MeRIP-RT-qPCR, it is unclear why changes in IP over input
308 sequencing reads are undetectable at the peaks reported by Bertero et al. (2018) and Huang et al. (2019)
309 but differences in peaks were successfully validated using MeRIP-RT-qPCR (22,65). While MeRIP-RT-
310 qPCR can be used as an initial method of validation for predicted peak changes, additional methods are
311 necessary to confirm quantitative differences in m^6A levels and to resolve points where the assays do not
312 agree.

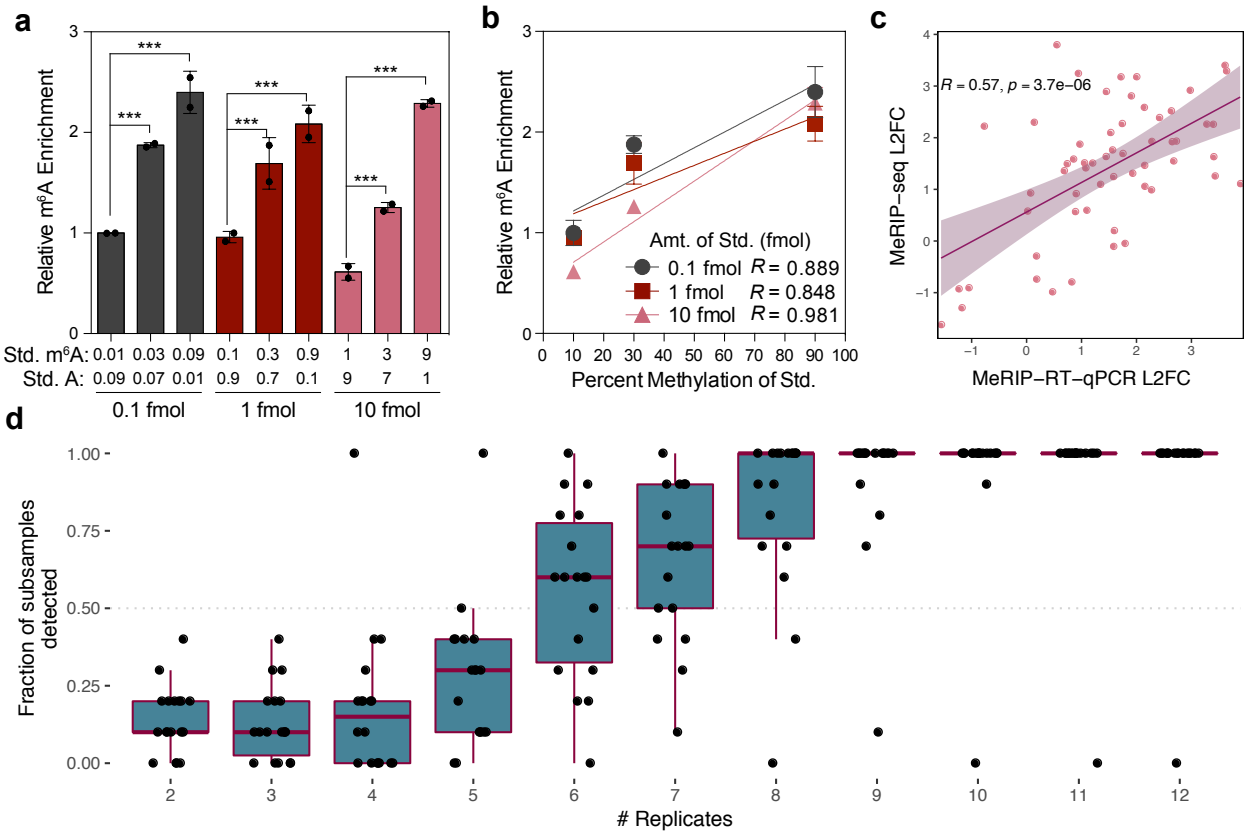

Figure 4: MeRIP-RT-qPCR validation and replicates necessary for the detection of peak changes. a) Relative enrichment of the indicated amounts of an in vitro transcribed standard containing unmodified A or m^6A, as measured by MeRIP-RT-qPCR. Data are shown for two independent replicates of three technical replicates each as IP enrichment over input relative to pulldown of a positive control spike-in, with the 0.1 fmol (0.01 m^6A: 0.09 A) sample normalized to 1. Bars represent mean ± SEM of two independent replicates. *** $p \leq 0.005$ by unpaired Student's *t*-test. **b)** Linear regression of relative m^6A enrichment from (a). Points and error bars mark mean ± SEM of two independent replicates. **c)** Change in MeRIP-RT-qPCR vs. MeRIP-seq enrichment for peaks detected as significantly differentially expressed with infection of Huh7 cells by dengue virus, Zika virus, and hepatitis C virus. **d)** Number of replicates of infected vs. uninfected cells needed to detect the peaks in (c). Replicates were randomly subsampled 10 times to calculate the fraction of subsamples in which peaks were called as significant by the GLMs or QNB. Boxes span the 1st to 3rd quartiles, with medians indicated. Whiskers show the minimum and maximum points within ±1.5x the interquartile distance from the boxes. Results for each subsample of replicates are shown as jittered points.

We next used our peaks validated using MeRIP-RT-qPCR to estimate the number of replicates necessary for detection of changes with either the GLM or QNB methods. Using a permutation test, we downsampled infected and uninfected replicates and reran statistical detection of changes. We found that approximately 6-9 replicates were necessary for consistent detection (in at least 50% of subsamples) of

317 most peak changes (**Figure 4d**), suggesting that almost all published MeRIP-seq studies to date are
318 underpowered.
319
320 **Discussion**
321 In the seven years since MeRIP-/m^{6}A-seq was first published (16,17), many studies have used
322 these methods to examine the function of m^{6}A, its distribution along mRNA transcripts, and how it might
323 be regulated under various conditions. While 35 out of 64 MeRIP- and miCLIP-seq papers we surveyed
324 **(Supplementary Table 1)** refer to m^{6}A as "dynamic", and, by contrast, only two describe the modification
325 as "static", the literature is unclear on what is meant by the word "dynamic". There is mixed evidence as to
326 whether m^{6}A is reversible through demethylation by FTO and ALKBH5 (66,71–73). While m^{6}A does not
327 appear to change over the course of an mRNA's lifetime at steady-state (3), whether it changes in
328 response to a particular stimulus and at what point is less clear. Some studies have suggested that m^{6}A
329 may be modulated through changes in methyltransferase and demethylase expression, producing
330 consistent directions of change across transcripts (8,23,34), through alternative mechanisms involving
331 microRNA, transcription factors, promoters, or histone marks (21,22,62,65,74), or through indeterminate
332 mechanisms (17,20,25–28,50). However, based on our reanalysis of available MeRIP-seq data, there is
333 still only meagre support for widespread changes in m^{6}A independent of changes in the expression of
334 methylation machinery (e.g. increases or decreases in METTL3 expression).
335 In particular, replication of peaks and changes in peaks across studies is limited. As with other
336 RNA IP-based methods, MeRIP-seq data contains noise, owing to technical and biological variation (75).
337 In fact, while peak overlaps reach ~80% between replicates of the same study, they decrease to a median
338 of 45% between studies, most of which use 2-3 replicates each **(Figure 1)**. Given that the detection of
339 peaks is so variable and that peak heights differ among replicates, it is perhaps not surprising that peak
340 changes have yet to be reproduced between multiple studies of similar conditions. Indeed, variability in
341 MeRIP-seq could also mask differences in m^{6}A regulation among cell types, which have been described
342 in mouse brains (34) and in cell lines exposed to KSHV (28). To distinguish biological and technical
343 variation, it will therefore be particularly important to test if multiple groups using the same cell line and
344 conditions can better reproduce changes in m^{6}A.
345 Disparities in the methods used to detect changes in m^{6}A$_{(m)}$ peaks also play a role in differing
346 conclusions among studies. Here, we analyzed four statistical methods to detect changes in peaks and
347 found that three of these methods showed uniform or conservatively shifted p-value distributions and were
348 able to identify changes in m^{6}A$_{(m)}$ independent of changes in gene expression. We therefore suggest that
349 these statistical methods, in combination with filters for input levels in both conditions and the difference in
350 $\log_2$ fold change between peaks and genes, can be used to identify candidate m^{6}A$_{(m)}$ sites from MeRIP-
351 seq data for further analysis and validation (**Figure 5)**. Based on our results, we do not recommend
352 MeTDiff for the detection of peak changes as it does not control well for differences in gene expression

353 **(Figure 2)**. Similar to others (33), we found that plotting predicted m^6A changes was invaluable with
354 appropriate scaling for gene coverage to reveal changes proportional to gene expression. In addition,
355 plotting the standard deviation in transcript coverage can help assess typical variation in peak height
356 among replicates. We note that both differential methylation of a gene and methylation of a gene that is
357 differentially expressed could be important, but they should not be conflated when considering the role of
358 m^6A in transcript regulation.

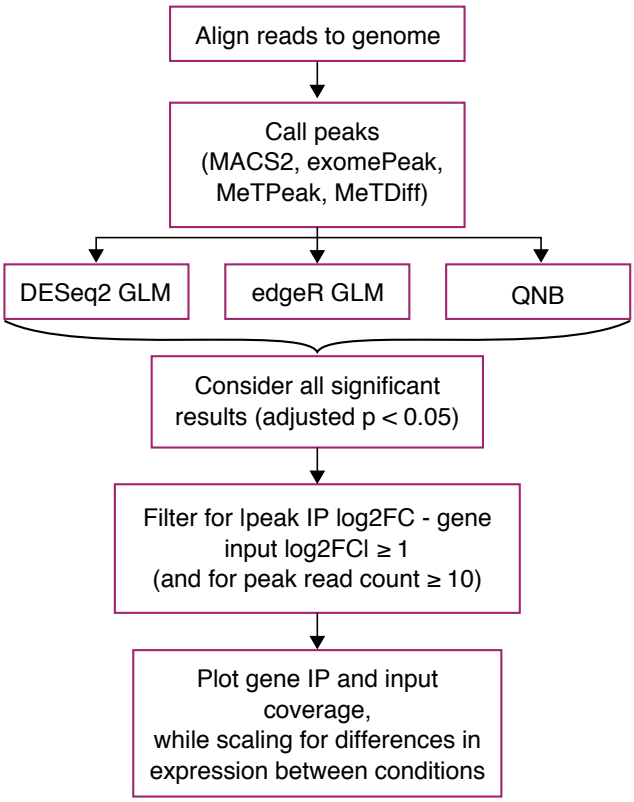

Figure 5: Proposed approach to identify candidates for $m^6A_{(m)}$ changes for further validation using MeRIP-seq data.

359

360 The extent to which m^6A changes on particular transcripts and whether it changes in binary
361 presence/absence or in degree is unclear. Although we found that MeRIP-RT-qPCR could detect
362 differences in in vitro transcribed RNA methylation and that these changes correlated with differences in
363 MeRIP-seq enrichment, neither MeRIP-seq nor MeRIP-RT-qPCR can reveal the precise fraction of
364 transcript copies modified by m^6A. In general, antibody-based methods are subject to biases, including
365 from differences in binding efficiencies based on RNA structure and motif preferences (76). There is an
366 oft-cited but little used antibody-independent method for site-specific quantification of m^6A, site-specific
367 cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography
368 (SCARLET) (19). However, methods that can directly detect and quantify m^6A over the transcriptome are
369 still needed (e.g. direct RNA sequencing, which has not yet been shown to accurately detect m^6A across
370 a cellular transcriptome (46)). A recently developed endoribonuclease-based approach is promising but
371 limited to sites within DRAC motifs ending in ACA, which comprise only a third of known m^6A sites

372 (44,45). Thus, endoribonuclease digestion specific to unmodified strands may enable quantitative
373 analyses of changes in m^6A:A ratios at ACA motif sites (44), but for now, site-specific SCARLET is the
374 only option to biochemically validate proposed changes in m^6A at other motifs.
375
376 **Conclusions**
377 Our work reveals the limits of MeRIP-seq reproducibility for the detection of $m^6A_{(m)}$ and in
378 particular suggests caution when using MeRIP-seq for the detection of changes in $m^6A_{(m)}$. To increase
379 confidence in predicted changes in $m^6A_{(m)}$, we propose statistical approaches that account for differences
380 in gene expression between conditions and variability among replicates. These methods can be used to
381 gain insight into the regulation and function of $m^6A_{(m)}$ and to predict specific sites for validation before the
382 development of high-throughput alternatives to MeRIP-seq, and similar strategies may be applicable to
383 other types of RNA sequencing assay.
384
385 **Methods**
386 *New MeRIP-seq data*
387 *- Huh7 data*
388 Total RNA was extracted from Huh7 cells using Trizol (Thermo-Fisher). mRNA was purified from 200 µg
389 total RNA using the Dynabeads mRNA purification kit (Thermo-Fisher) and concentrated by ethanol
390 precipitation. Purified mRNA was fragmented using the RNA Fragmentation Reagent (Thermo-Fisher) for
391 15 minutes followed by ethanol precipitation. Then, MeRIP was performed using EpiMark N6-
392 methyladenosine Enrichment kit (NEB). 25 µL Protein G Dynabeads (Thermo-Fisher) per sample were
393 washed three times in MeRIP buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-40) and incubated
394 with 1 µL anti-m^6A antibody (NEB) for 2 hours at 4°C with rotation. After washing three times, anti-m^6A
395 conjugated beads were incubated with purified mRNA with rotation at 4°C overnight in 300 µL MeRIP
396 buffer with 1 µL RNAse inhibitor (recombinant RNasein; Promega). Beads were then washed twice with
397 500 µL MeRIP buffer, twice with low salt wash buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-
398 40), twice with high salt wash buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-40), and once
399 again with MeRIP buffer. m^6A-modified RNA was eluted twice in 100 µL MeRIP buffer containing 5mM
400 m^6A salt (Santa Cruz Biotechnology) for 30 minutes at 4°C with rotation and concentrated by ethanol
401 precipitation. RNA-seq libraries were prepared from eluate and the 10% of RNA set aside as input using
402 the TruSeq mRNA library prep kit (Illumina) and checked for fragment length using the Agilent 2100
403 Bioanalyzer. Single-end 50 base pair reads were sequenced on an Illumina HiSeq 2500.
404 *- Heat shock*
405 Early passage OCI-Ly1 diffuse large B-cell lymphoma cells were grown in Iscove's modified Eagle
406 Medium (IMDM) with 10% fetal bovine serum (FBS). OCI-Ly1 cells were obtained from the Ontario
407 Cancer Institute and regularly tested for *Mycoplasma* contamination by PCR and identified by single

408 nucleotide polymorphism. Cells were maintained with 1% penicillin/streptomycin in a 37°C, 5% CO_2,
409 humidified incubator. In these growing conditions, heat shocked cells were exposed to 43 °C for 1 hour,
410 followed by 1 hour of recovery at 37°C while control cells were maintained at 37°C. Following treatment,
411 cells were processed at 4°C to obtain total cell lysates. Lysates were immunoprecipitated for $m^6A_{(m)}$ using
412 Synaptic Systems antibody (SYSY 202 003) following the protocol described in Meyer, et al (2012) and
413 sequenced on an Illumina HiSeq 2500 (16).
414
415 *Read processing*
416 Reads were trimmed using Trimmomatic (77) and aligned to the human genome (hg38) or the mouse
417 genome (mm10), as appropriate, using STAR (67).
418
419 *Peak detection and comparison*
420 IP over input peaks were called using MACS2 (49). Transcript coverage was estimated using Kallisto (78)
421 with an index construct 31mers, except for the Schwartz et al (2014) data set, where the reads were too
422 short and an alternative index based on 29mers was constructed (33). For **Figure 1b**, the full union of
423 unique peaks was taken and the percent of that set detected in single replicates calculated. Intersects
424 between peaks that overlapped for transcripts with ≥10X mean coverage in both samples were taken
425 using bedtools (79) for **Figure 1c-d**, allowing a generous minimum of 1 overlapping base. Heatmaps for
426 peak overlaps were generated using the ComplexHeatmap package in R (80). MeRIP-seq data sets in
427 **Figure 1d** included those for human cell lines in **Figure 1c**, other data sets from the same studies and
428 any data sets that shared the same cell lines, and other data sets that looked at multiple human cell
429 types. We considered only data sets from baseline conditions in **Figure 1** (untreated cells and knockdown
430 controls).
431
432 *Poisson and negative binomial fits*
433 Poisson and negative binomial models were fit to input and IP read counts at peaks using maximum
434 likelihood estimation. Simulated read counts were generated with Poisson or negative binomial
435 distributions based on estimated parameters from the sample, with 500 random generations per model.
436 The log likelihood of seeing read counts from the sample and the simulations given the model parameters
437 was then calculated and the mean taken across all peaks.
438
439 *Peak change detection and generalized linear models*
440 Generalized linear models to detect changes in IP coverage while controlling for differences in input
441 coverage were implemented based on a method previously applied to HITS-CLIP data (54). Full and
442 reduced models were constructed as follows:
443

$$\log \mu_{ij} = \beta_i^0 + \beta_i^{IP} X_j^{IP} + \beta_i^{STIM} X_j^{STIM} + \beta_i^{STIM:IP} X_j^{STIM:IP}$$

$$\log \mu_{ij} = \beta_i^0 + \beta_i^{IP} X_j^{IP} + \beta_i^{STIM} X_j^{STIM}$$

Where μ_{ij} is the expected read count for peak i in sample j, modelled as a negative binomial distribution, $X_j^{IP} = 1$ for IP samples and 0 for input samples, and $X_j^{STIM} = 1$ for samples under the experimental intervention and 0 for control samples.

Statistical significance was then assessed using a chi-squared test (df=1) for the difference in deviances between the full and reduced models, with the null hypothesis that the interaction term ($\beta_i^{STIM:IP}$) for differential antibody enrichment driven by the experimental intervention is zero. The likelihood ratio test was implemented through DESeq2 (52) and edgeR (53), two programs developed for RNA-seq analysis that differ in how they filter data and in how they estimate dispersions for negative binomial distributions. Generalized linear models implemented through edgeR included a term for the normalized library size of sample j.

QNB was run as suggested for experiments with biological replicates, where each IP and input variable ("ip1", etc.) consisted of a matrix of peak counts for either condition 1 or condition 2:

```
> qnbtest(ip1, ip2, input1, input2, mode="per-condition")
```

We extracted functions from MeTDiff so that we could supply our own peaks and thus control for differences in peak detection among tools. The main post-peak calling function, diff.call.module, was run as follows using the same count matrices as for QNB:

```
>  diff.call.module(ip1, input1, ip2, input2)
```

Gene and peak expression changes were estimated as $\log_2$ fold changes from DESeq2 based on differences in input read counts aligned to genes and IP read counts aligned to peaks, respectively, and the change in peak relative to gene enrichment was calculated as the absolute difference in $\log_2$ fold change between those values.

Comparison to published studies

We selected data sets for reanalysis in **Figure 3a** based on the availability of ≥ 2 replicates and a published estimate of the number of m^6A changes between two conditions. The sources for published estimates of m^6A peak changes included in our comparison are listed in **Supplementary Table 4**. Significant (FDR-adjusted $p < 0.05$) peaks were considered for DESeq2, edgeR, and QNB, run as described above. We also considered a filtered set of peaks derived from the union of significant peaks from the three tools with additional filters for location within exons, $|\log_2$ fold change between peak IP and

480 gene inputl ≥ 1, and a minimum peak read count of 10 across replicates and conditions. We used
481 gProfiler to calculate enrichment of functional categories using a hypergeometric test (81).
482
483 In **Figure 3b-c**, we selected *Hspa1a/HSPA1A* as our representative gene for heat shock because it was
484 the primary example cited by Zhou et al. (2015) and Meyer et al. (2015) (4,20). For HIV, we selected
485 *PSIP1* because it was among the 56 genes reported by Lichinchi et al. (2016a) (25), it plays a known role
486 in HIV infection, and we detected a peak in the gene using MACS2.
487
488 For KSHV, we compared significant results (adjusted $p < 0.05$) from QNB and GLMs (DESeq2 and
489 edgeR), with additional filtering for lpeak IP – gene input $\log_2$ fold changel ≥ 1 (lowering this threshold to
490 0.5 did not change results), for data from Hesser et al. (2018) (27) in lytic vs. latent iSLK.219 cells and
491 data from Tan et al. (2018) (28) in lytic vs. latent iSLK BAC16 cells. We used the same approach to
492 compare data from Rubio et al. (2018) and Winkler et al. (2019) (69,70) for response to dsDNA. Data sets
493 used for site-specific comparisons are summarized in **Supplementary Table 5**.
494
495 Gene coverage was plotted using CovFuzze (https://github.com/al-mcintyre/CovFuzze), which
496 summarizes mean and standard deviation in coverage across available replicates.
497
498 *Spike-in controls and MeRIP-RT-qPCR*
499 In vitro transcribed (IVT) controls were provided by the Jaffrey Lab and consisted of 1001 base long RNA
500 sequences with three adenines in GAC motifs (**Supplementary Table 6**) either fully methylated or
501 unmethylated. m^6A and A controls were mixed in various ratios (1:9, 3:7, and 9:1) that approximate the
502 variation in m^6A levels detected by SCARLET (m^6A levels at specific sites have been reported to vary
503 from 6-80% of transcripts (19)). Modified and unmodified standards were mixed at the indicated ratios to
504 yield a final quantity of 0.1 fmol, 1 fmol, and 10 fmol. Mixed RNA standards were added to 30 µg total
505 RNA from Huh7 cells, along with 0.1 fmol of positive (m^6A-modified *Gaussia* luciferase RNA, "GLuc") and
506 negative control (unmodified *Cypridina* luciferase, "CLuc") spike-in RNA provided with the N6-
507 methyladenosine Enrichment kit (EpiMark). Following MeRIP as described above, cDNA was synthesized
508 from eluate and input samples using the iScript cDNA synthesis kit (Bio-Rad), and RT-qPCR was
509 performed on a QuantStudio Flex 6 instrument. Data was analyzed as a percent of input of the spike-in
510 RNA in each condition relative to that of the provided positive control spike-in. Primers used for RT-qPCR
511 were:
512 IVT_Std_F: TGCCTTTTCTTTCGGTTGCG
513 IVT_Std_R: CAAACACAAGAAGGCACGGG
514 GLuc_F: CGACATTCCTGAGATTCCTGG
515 GLuc_R: TTGAGCAGGTCAGAACACTG

516 CLuc_F: GCTTCAACATCACCGTCATTG
517 CLuc_R: CACAGAGGCCAGAGATCATTC
518
519 *Cell culture and infection (data used for MeRIP-RT-qPCR experiments)*
520 Huh7 cells were grown in DMEM (Mediatech) supplemented with 10% fetal bovine serum (HyClone), 2.5
521 mM HEPES, and 1X non-essential amino acids (Thermo-Fisher). The identity of the Huh7 cell lines was
522 verified using the Promega GenePrint STR kit (DNA Analysis Facility, Duke University), and cells were
523 verified as mycoplasma free by the LookOut Mycoplasma PCR detection kit (Sigma). Infectious stocks of
524 a cell culture-adapted strain of genotype 2A JFH1 HCV were generated and titered on Huh7.5 cells by
525 focus-forming assay (FFA), as described (82). Dengue virus (DENV2-NGC), West Nile virus (WNV-
526 NY2000), and Zika virus (ZIKV-PRVABC59) viral stocks were generated in C6/36 cells and titered on
527 Vero cells as described (82). All viral infections were performed at a multiplicity of infection of 1 for 48
528 hours.
529
530 **Availability of Data and Materials**

531 MeRIP-seq data for the Huh7 negative controls is available in the GEO repository, under accession
532 number GSE130891. MeRIP-seq data for heat shock in B-cell lymphoma is available under accession
533 number GSE130892. Accession numbers for all other data sets reanalyzed in the study are included in
534 Supplementary Tables 1-5. Scripts used for analyses are available at https://github.com/al-
535 mcintyre/merip_reanalysis_scripts.

536 **Competing interests**

537 C.E.M. is a cofounder and board member for Biotia and Onegevity Health, as well as an advisor or
538 compensated speaker for Abbvie, Acuamark Diagnostics, ArcBio, BioRad, DNA Genotek, Genialis,
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548 **Authors' contributions**

549 A.B.R.M. and C.E.M. conceived the study. A.B.R.M. developed and ran the analyses and wrote the
550 manuscript with N.S.G. and S.M.H. S.R.J. provided in vitro controls for MeRIP-RT-qPCR. N.S.G. prepared
551 MeRIP-seq libraries for the Huh7 controls and ran MeRIP-RT-qPCR tests. L.C. contributed additional heat
552 shock data. All authors read and edited the manuscript.

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