Enzymes flying under the radar: Cryptic METTL3 can persist in knockout cells

Sigrid Nachtergaele

Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut, United States of America

* sigrid.nachtergaele@yale.edu

The presence of m^6A in mRNA of METTL3 knockout cells has long been a point of confusion. In this issue of PLOS Biology, Poh and colleagues reveal alternatively spliced, catalytically active METTL3 isoforms that persist in cells previously thought to lack the enzyme.

RNA molecules are modified with over 160 chemically diverse groups that can tune RNA structure, stability, and function. While RNA modifications were first studied primarily in abundant noncoding RNAs, we now know that mRNA also carries several of these chemical marks [1]. In particular, the discovery and characterization of N^6-methyladenosine (m^6A) in mRNA over the last decade has led to an explosion of work demonstrating its function in a variety of organisms and cell types [2]. This methyl mark is installed on mRNA by the METTL3-METTL14 complex [3] and can regulate mRNA splicing, export, translation, and decay. However, as numerous groups sought to dissect m^6A function and regulation, they ran into the puzzling result that mRNA m^6A persisted in some cell lines despite apparent complete knockout of the catalytic component METTL3.

For instance, 2 studies independently generated Mettl3 knockout mouse embryonic stem cells (mESCs) and found loss of approximately 60% and approximately 99% of mRNA m^6A, respectively [4,5]. The first study used CRISPR-Cas9-mediated knockout with a guide RNA targeting exon 2 (exon2 Mettl3 KO mESC) [4], while the second used Cre-Lox recombination to excise exon 4 (exon4 Mettl3 KO mESC) [5]. At the time, it was thought that the METTL3 binding partner, METTL14, might have sufficient m^6A methyltransferase activity to compensate for METTL3 loss. This has since been shown not to be the case, resulting in the hypothesis that another yet-to-be identified methyltransferase was responsible for the remaining m^6A.

While additional m^6A methyltransferases, such as METTL16 [6] and METTL5 [7], have since been identified, their activity on mRNA is thought to be limited. Even if these enzymes could partially compensate for METTL3 loss, how this could result in such high variability in the amount of remaining m^6A was mysterious. Genetic knockout of Mettl3 represented a powerful tool to study the role of m^6A in a variety of biological systems. But the fact that the modification persisted to such different degrees was a significant complication in interpreting these experiments and called into question whether the observed phenotypes were, indeed, the result of loss of m^6A in mRNA.

Poh and colleagues [8] set out to settle this critical question through comprehensive characterization of the cells in question, including exon2 Mettl3 KO mESC and exon4 Mettl3 KO
mESC. These cell lines were generated when research on m6A in mRNA was in its infancy, so each study used different reagents and methods to measure m6A modification and METTL3 protein levels. To ensure that technical differences were not to blame, m6A abundance in mRNA was re-analyzed by mass spectrometry, confirming the dramatic differences described previously. However, careful examination of western blots using 2 different anti-METTL3 antibodies revealed the presence of additional METTL3 species that varied across cell lines.

The fact that exon2 Mettl3 KO mESCs had significantly more residual m6A than exon4 Mettl3 KO mESCs, combined with the possible presence of alternative METTL3 protein isoforms, suggested that the different methods and genomic locations used for mutagenesis may have resulted in alternatively spliced transcripts (Fig 1). Of these transcripts, some could have translation start sites that would allow for catalytically active METTL3 isoforms to be translated. To test this hypothesis, Poh and colleagues first used 5′ RACE to identify which alternatively spliced Mettl3 transcripts were present in each cell line. Using this information, they determined which METTL3 isoforms predicted to be translated from these transcripts had m6A methyltransferase activity. Polysome profiling then confirmed that these alternatively spliced Mettl3 transcripts were indeed translated in cells. As a secondary demonstration that isoforms of METTL3 itself, and not another methyltransferase, were responsible for the residual m6A, Poh and colleagues took advantage of a recently developed METTL3 inhibitor,

![Fig 1.](https://doi.org/10.1371/journal.pbio.3001717.g001)
STM2457 [9]. Treatment of exon2 Mettl3 KO mESC with this inhibitor significantly reduced mRNA m6A levels, indicating that the origin of this m6A was indeed a METTL3 isoform, as STM2457 does not inhibit other RNA methyltransferases.

While it remains possible that small numbers of mRNA m6A sites are installed by other RNA methyltransferases, the multiple orthogonal approaches taken in this work strongly suggest that the majority of this modification is installed by the METTL3-METTL14 complex. This work also provides new clarity on the question of whether Mettl3 is an essential gene. Mettl3 knock out in mice is embryonic lethal, and genetic screens in human cell lines suggest that they also do not survive METTL3 loss. Delving into the DepMap dataset [10], Poh and colleagues found that the majority of cell lines were, indeed, dependent on METTL3 for survival. The fact that stable, viable METTL3 knockout human cell lines could be generated at all was therefore a paradox, but it now appears likely that many of these lines survived by relying on alternatively spliced METTL3 isoforms. Taken together, this study provides an elegant answer to a question that has plagued the field since METTL3 knockout cell lines and organisms were first generated. Moreover, it provides a framework within which researchers can now assess their own reagents and results.

More broadly, however, it should also serve as a cautionary tale. CRISPR-Cas9 has revolutionized our ability to quickly generate knockout cell lines for almost any gene of interest. But, as this work reveals, the details of its implementation can dramatically impact the results. The analysis by Poh and colleagues demonstrated that a smaller mutation in an earlier exon was insufficient to eliminate METTL3 activity, while excision of a later exon resulted in a true genetic knockout [8]. In this case, a combination of alternative splicing and the ability of individual protein domains to form stable folds came together to produce sufficient cryptic METTL3 activity to install significant amounts of m6A on mRNA. However, these 2 processes, alternative splicing and stable folding of protein domains, are of course ubiquitous in biology. Thus, it is likely that functionally incomplete knockout cell lines are more common than we realize. Moving forward, proactively thinking about possible alternative isoforms when designing knockout strategies, as well as more detailed characterization of the resulting knockout cell lines, will be critical to ensure correct data interpretation.

References
1. Martinez NM, Gilbert WV. Pre-mRNA modifications and their role in nuclear processing. Quant Biol. 2018; 6(3):210–27. Epub 2018/12/12. https://doi.org/10.1007/s40484-018-0147-4 PMID: 30533247; PubMed Central PMCID: PMC6284822.
2. Meyer KD, Jaffrey SR. Rethinking m(6)A Readers, Writers, and Erasers. Annu Rev Cell Dev Biol. 2017; 33:319–42. Epub 2017/08/02. https://doi.org/10.1146/annurev-cellbio-100616-060758 PMID: 28759256; PubMed Central PMCID: PMC5963928.
3. Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, et al. A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. Nat Chem Biol. 2014; 10(2):93–5. https://doi.org/10.1038/nchembio.1432 PMID: 24316715; PubMed Central PMCID: PMC3911877.
4. Batista PJ, Molnie B, Wang J, Qu K, Zhang J, Li L, et al. m(6)A RNA modification controls cell fate transition in mammalian embryonic stem cells. Cell Stem Cell. 2014; 15(6):707–19. https://doi.org/10.1016/j.stem.2014.09.019 PMID: 25456834; PubMed Central PMCID: PMC4278749.
5. Geula S, Moshtch-Moskowitz S, Dominissini D, Mansour AA, Kol N, Salmon-Divon M, et al. Stem cells. m6A mRNA methylation facilitates resolution of naïve pluripotency toward differentiation. Science. 2015; 347(6225):1002–6. https://doi.org/10.1126/science.1261417 PMID: 25569111.
6. Pendleton KE, Chen B, Liu K, Hunter OV, Xie Y, Tu BP, et al. The U6 snRNA m(6)A Methyltransferase METTL16 Regulates SAM Synthetase Intron Retention. Cell. 2017; 169(5):824–35 e14. https://doi.org/10.1016/j.cell.2017.05.003 PMID: 28525753; PubMed Central PMCID: PMC55502809.
7. van Tran N, Ernst FGM, Hawley BR, Zorbas C, Ulyrcz N, Hackert P, et al. The human 18S rRNA m6A methyltransferase METTL5 is stabilized by TRMT112. Nucleic Acids Res. 2019; 47(15):7719–33. Epub 2019/07/23. https://doi.org/10.1093/nar/gkz619 PMID: 31328227; PubMed Central PMCID: PMC6735865.
8. Poh HX, Mirza AH, Pickering BF, Jaffrey SR. Alternative splicing of METTL3 explains METTL3-independent m6A in mRNA. PLoS Biol. 2022; 20(7):e3001683. https://doi.org/10.1371/journal.pbio.3001683

9. Yankova E, Blackaby W, Albertella M, Rak J, De Braekeleer E, Tsagkogeorga G, et al. Small-molecule inhibition of METTL3 as a strategy against myeloid leukaemia. Nature. 2021; 593(7860):597–601. Epub 2021/04/27. https://doi.org/10.1038/s41586-021-03536-w PMID: 33902106.

10. DepMap. DepMap 21Q2 Public. In: figshare, editor. 2021.