The m6A reader YTHDF2 and m1A eraser ALKBH3 fine-tune mRNA transgene expression in CHO cells

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Abstract:

N6-methylated adenosine (m6A) and N1-methylated adenosine (m1A) are two epi-transcriptomic modifications on eukaryotic mRNA which have recently been rediscovered and are generating considerable interest. M6A methylation impacts on all aspects of cellular RNA metabolism and numerous physiological processes. Although less abundant than the m6A epitranscriptomic mark, m1A methylation has recently also attracted interest due to its dynamic nature in response to physiological changes. We investigated the role of the m6A and m1A methylation regulators on the expression of a transgene in Chinese Hamster Ovary (CHO) cells - the host cell of choice in producing biopharmaceutical proteins commercially. Using siRNA-mediated gene depletion and methylation-specific RNA immunoprecipitation with anti-m6A or m1A-antibodies, we show that (i) knock-down of the m6A 'reader' YTHDF2 or the m1A 'eraser' ALKBH3 dramatically impacts transgene expression; (ii) the effects of YTHDF2 and ALKBH3 depletion on transgene expression are m6A- and m1A-mediated. We conclude that the expression of transgenes in CHO cells can be subjected to regulation by both m6A and m1A regulators. These findings open up the prospect of previously unexplored epi-transcriptomic-based approaches to CHO cell line engineering for improved recombinant protein production.

Key words: CHO cells, transgene expression, m6A reader YTHDF2, m1A eraser ALKBH3

1 Introduction

A large number of studies in recent years have convincingly proven the impact of N^6-methyl adenosine (m6A) - the most abundant modification on eukaryotic mRNA - on all aspects of RNA metabolism including spicing, stability, translation, microRNA processing (Alarcón, Lee, Goodarzi, Halberg, & Tavazoie, 2015; Dominissini et al., 2012; Han et al., 2015; Meyer et al., 2015; Roundtree & He, 2016; Wang et al., 2014; Zhou et al., 2015) and several physiological processes including cancer, immunity and memory (Lu et al., 2018; Müller et al., 2018; Wang et al., 2019). The reversible m6A modification is installed by the m6A writer complex (Knuckles & Bühler, 2018) containing the catalytic core METTL3 and METTL14 proteins (Liu et al., 2014) and removed by the m6A erasers ALKBH5 and FTO (Ensfelder et al., 2018; Wei et al., 2018). The m6A readers bind m6A RNA targets to mediate their fate. At present, more than twenty m6A readers have been identified (Edupuganti et al., 2017; Huang et al., 2018; Wu et al., 2018), of which the 5-member YT521-B Homology (YTH) domain-containing protein reader family (YTHDF1-3, YTHDC1, and YTHDC2) that directly bind m6A through the YTH domain, are the most studied. (Patil, Pickering, & Jaffrey, 2018). Notably, the three cytoplasmic YTHDF members (YHTDF1-3 or DF1-3 for short) have been shown to act in an integrated and coordinated manner in affecting the fate of their shared m6A RNA targets and ultimately protein expression. Cooperation between DF1 and DF3 has been shown to promote translation, whereas DF2 and DF3 conspire to accelerate m6A-containing RNA decay (Shi et al., 2017; Zhao et al., 2017).

N^1-methyl adenosine (m1A) is another epigenetic mark on mRNA that has recently attracted interest due to the observation that it is dynamically regulated in response to changes in cellular or physiological conditions, and is generally positively correlated with protein expression ((Dominissini et al., 2016; Yi et al., 2016). The tRNA methyltransferase complex TRMT6/61A has been implicated in m1A modification of some nuclear mRNAs at m1A sites with t-RNA T-loop-like structures (Li et al., 2017; Safra et al., 2017) however, for the majority of m1A sites in mRNA, the responsible m1A writers are yet to be identified. ALKBH3 - also known as prostate cancer antigen-1 (PCA-1) has been shown to be an m1A eraser in mammalian cells (Li et al., 2017; Safra et al., 2017).

Given the abundance/significance of these modifications in mRNA, we were interested in establishing if the expression of a transgene could be impacted by m6A and m1A regulation in Chinese Hamster Ovary (CHO) cells - the dominant cell line for commercial production of biopharmaceutical proteins. We reasoned that productivity in CHO cells might be impacted by modulating the regulators of these epitranscriptomic marks. However, knowing with certainty if a transgene is subjected to m6A or m1A regulation remains a challenge at present, as m6A methylation also occurs in sequences outside the canonical DRACH methylation motif (D=A/G/U, R=A/G, A=m6A, and H=A/C/U), and not all DRACH motifs are subjected to m6A methylation (Linder et al., 2015). In addition, a universal method to precisely identify m1A in mRNA is not yet available (Chen, Feng, Tang, Ding, & Lin, 2016). We chose Green Fluorescence Protein (GFP) as a model reporter transgene.

2 Materials and methods

2.1 Cell lines and reagents.

CHO-K1 cell line (ATCC® CCL-61) was adapted for suspension culture and routinely tested for mycoplasma contamination. Cells were maintained in Serum-Free Media (SFM) supplemented with 2.52 g/L anti-clumping polyvinyl alcohol (PVA) unless otherwise stated. Cultures were grown in a Climo-Shaker (Kühner) at 37 °C, 80% humidity and 5% CO_2, 170 rpm. GFP and both cell growth and viability were monitored using the Guava Express Plus and Guava Pro programmes, or Accuri BD Sampler Plus, respectively on the Guava EasyCyte (Merck Millipore) or the BD Accuri™ C6 Plus system (Becton, Dickinson and Company BD Biosciences). Reagents are listed in Table S1A.

2.2 Vectors

The vectors CMV-d2GFP-HYG and CMV-EPO-HYG which contains a coding sequence for the unstable GFP (d2GFP) or Erythropoietin (EPO) between the CMV promoter and polyA, were used in this work. Clone OHu31338D containing the open reading frame of the human ALKBH3 in pcDNA3.1 with C-terminal FLAG tag was obtained from GenScript, U.S.A. The overexpression vector CMV-DF2 was constructed as follows: The open reading frame (ORF) of DF2 (GenBank accession number XM_007626785) was first amplified using cDNA made from DNA-free total RNA

from CHO-K1 as a template and primer pair DF2for and DF2rev. The resulting amplicon was used as a template to incorporate 3 FLAG epitopes upstream of the start codon using overlapping forward primers (FLAG2YN and 3FLAGBamfor) and reverse primer Y2endXho. The resulting fragment was cloned into pcDNA3 at the BamHI and XhoI sites. Primer sequences are listed in Table S1B.

2.3 Transfection

Transfection was carried out in a 24 well suspension plate using TransIT-X2® transfection reagent, following the protocol recommended by the manufacturer (Mĭrus). For co-transfection of siRNA and plasmid DNA, the siRNA was transfected 16-24 hours in advance of the plasmids. The final concentration of siRNA was 25 nM and 400 ng DNA plasmid per 1 x 10^6 cells/mL. SiRNA was designed and supplied by Integrated DNA Technologies (IDT), including the siRNA negative control (NC). SiRNA sequences are listed in Table S1C.

2.4 RT-PCR

cDNA was prepared using High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol. RT-PCR was performed using FAST SYBR kit with three technical replicates and analyzed on an Applied Biosystems 7500 FAST and Real-Time PCR system. The fold change of gene expression was calculated using the 2^(-ΔΔCt) method. Primers used in qPCR are listed in Table S1D. HPRT1 was used as a reference gene.

2.5 Western blot analysis

Cells were lysed in RIPA buffer containing Halt™ protease inhibitor cocktail at 4 ºC for 1 hour with gentle rotation. Cell debris was removed by centrifugation at 14,324 x g for 10 min, protein lysate was heated at 70 ºC for 10 min in the presence of 1X loading buffer. Proteins were separated on a precast 4-12% Bis-Tris Plus gel in Bolt™ MOPS SDS running buffer. Gels were blotted on a Nitrocellulose membrane using Thermo Scientific Pierce Power Blotter (# 22834) in Pierce 1 step transfer buffer. Blots were scanned and quantitatively analyzed using the Odyssey ® Fc Imaging System.

2.6 M6A and m1A immunoprecipitation.

M6A and m1A immunoprecipitation were adapted from the protocols provided in the EpiMark® N6-Methyladenosine Enrichment Kit (NEB #E1610S, New England BioLabs Inc). Briefly, 3.5 ug enriched mRNA or 3 ug fragmented, enriched mRNA (90 ºC for 5min in NEB fragmentation buffer (NEB *#E6150S*)) was incubated for 3 hours with Protein A/G agarose beads that were pre-incubated with 5 ug of an anti-m6A, -m1A or -Gapdh antibody for 1 hour with head to tail rotation in reaction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-40). Beads were washed twice with reaction buffer, twice in a low salt buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-40) and twice in a high salt buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% NP-40). Immunoprecipitated RNA was eluted using a reaction buffer containing 6.7 mM N6-methyladenosine or 3 mg per mL N1-methyladenosine. All buffers contained an RNase inhibitor. 350 uL of eluted RNA was precipitated with 2.5 volume of absolute ethanol, 0.3M NaAc, pH 5.5 and 1.5 uL glycogen overnight at -80 ºC, washed twice in 75% Ethanol and resuspended in 15 uL water. cDNA synthesis and qPCR were carried out as described in EpiMark® N6-Methyladenosine Enrichment Kit using High-Capacity cDNA synthesis and Fast SYBR™ Green Master Mix kits. The percentage of immunoprecipitate (IP) to Input was calculated as described in the Magna MeRIPTM m6A kit (17-10499, Millipore). mRNA controls (m6A RNA and unmodified RNA) were used as described in the EpiMark® kit.

3 Results and discussion

3.1 GFP expression is affected by both m6A and m1A regulators

First, we found that there were no m6A sites predicted in the GFP transcript using on-line prediction software (http://www.cuilab.cn/sramp/) (Zhou, Zeng, Li, Zhang, & Cui, 2016) which is based on the presence of the DRACH consensus motif. However, there are 25 GAC motifs, which have been shown to be a preferred target for the m6A reader DF1/2 (Wang et al., 2014), two of which are GACT. In addition, we found two predicted sites for m1A within the coding sequence of GFP, one of which (GTTCGA) was previously identified by two studies mapping m1A occurrence in human cells (Li et al., 2017; Safra et al., 2017) and a second putative m1A site which is GA rich (GGAAGA) identified by antibody-dependent and independent mapping approaches (Yi et al., 2016; Zhou et al.,

2019). We characterized the expression of GFP in CHO-K1 cells co-transfected with siRNA targeting METTL3, METTL14, a regulator of writer complex formation (WTAP), m6A erasers and the m1A eraser ALKBH3. We found that the level of GFP protein expression was substantially reduced when the m6A readers DF1 or DF3 were silenced, as opposed to silencing DF2 which increased GFP expression nearly 2-fold (Figure 1A). The relative levels of GFP mRNA were 4-fold higher in DF2-silenced cells compared to that of the siNC suggesting that the GFP mRNA was more stable when DF2 was depleted (Figure 1B), consistent with DF2's putative role in accelerating mRNA degradation (Wang et al., 2014). The expression of GFP at the transcript level showed significant differences upon knock-down of different readers/writers, nearly two-fold greater than the variation at the protein level. This is not unexpected, as these readers are known to impact mRNA abundance by various mechanisms outlined above, and as other m6A readers, of which more than 20 have been identified may also be involved. The expression of GFP was reduced by nearly 40% in siALKBH3 transfected cells while only being negligibly affected when other m6A regulators (METTL3, METTL14, and WTAP, ALKBH5 and FTO) were depleted (Figure 1C, D, E). Cell viability and growth were not affected (Figure S1). One might expect knocking down METTL3 to achieve the same effect as depleting DF2, however this assumes that the absence of an m6A modification is the same as the absence of a reader that destabilises an m6A-modified target mRNA. Suppressing methylation of a transcript by METTL3-depletion, while preventing DF2 binding, may also prevent other translation-promoting factors to bind the mRNA.

3.2 Interplay between m6A reader YTHDF2 and m1A eraser ALKBH3 in the regulation of GFP expression

It has been suggested that members of the DF sub-family and YTHDC1 can bind directly to m1A in RNA. In particular, the conserved Trp^{432} in the YTH domain of DF2 (Figure S2), which is necessary for its binding to m6A, is required for its recognition of m1A (Dai, Wang, Gonzalez, & Wang, 2018). We characterized the expression of GFP in cells over-expressing DF2 and depleted for either ALKBH3, ALKBH5 or FTO (Figure 2 A, B). Over-expression of DF2 (CMV-DF2) reduced the expression of GFP in cells co-transfected with the control siNC by 10%. When co-transfected with

siALKBH3 or ALKBH5 however, the expression of GFP was further reduced (Figure 2C, D). This suggests that DF2, in addition to acting as an m6A reader, most likely also interacts with or influences the m1A target site of ALKBH3 on GFP, inducing further degradation of GFP. This is supported by the fact that the level of ALKBH3 was the same in cells co-transfected with siALKBH3 and CMV-DF2 or CMV-FLAG (Figure 2A), ruling out the impact of DF2 through further reducing the levels of ALKBH3, as ALKBH3 was found also to be subjected to negative regulation by DF2 (Figure S4B). The levels of GFP (protein and transcript) in CHO cells co-transfected with siFTO or ALKBH5 and CMV-FLAG were different, possibly due to the fact that FTO has been shown to act on other mRNA modifications including m1A of tRNA and m6Am in addition to m6A, and its RNA demethylation is context-dependent (Shi et al., 2019). This also suggests that not only is mRNA stability affected by these proteins but translational effects also come to play. Despite the impact of depleting ALKBH3 on GFP levels, over-expression of the human ALKBH3 (89% amino acid identity with its CHO counterpart) did not enhance the level of GFP produced (Figure S3). The levels of ALKBH3 protein found in different cell lines vary considerably, from undetectable to the same level as that of actin (Yi et al., 2016). It is likely that in CHO cells further increasing ALKBH3 protein levels has no further impact on its target genes, or perhaps the human homologue is less active in CHO cells.

In order to gain further insight into any potential synergy between the m6A and m1A modifications on GFP expression, we characterised the expression of GFP in cells depleted for both the m6A writer METTL3 and the m1A eraser ALKBH3. We found that there was no significant change in the level of GFP protein in cells with both proteins knocked down, compared to siALKBH3 alone, however knocking down both simultaneously had a slightly greater impact on GFP mRNA than targeting ALKBH3 alone, and similar to further depletion of ALKBH3 using high concentration siRNA. Neither had the depletion of m1A eraser ALKBH3 any impact on the level of METTL3 transcript, nor vice versa (Figure S3F, S3G and S4E).

3.3 Regulation of GFP mRNA by DF2 is m6A-mediated and localisation of m6A sites on GFP mRNA

To confirm that the regulation of GFP by DF2 is m6A-meditated we performed immunoprecipitation of m6A methylated mRNA from cells treated with siNC or siDF2 using an anti-m6A antibody. Both

positive and negative control mRNA behaved as expected (Figure S4A). In cells transfected with siRNA (NC or DF2) and GFP plasmid, GFP mRNA was enriched in the m6A_IP fraction compared with the Input (Figure 3A). Similarly, SON mRNA, which is known to be methylated (Wang et al., 2014), was successfully pulled down by the anti-m6A antibody. Interestingly, m6A writer complex METTL3, METTL4, WTAP, m6A DF1-3 readers and erasers, and the m1A eraser were also captured by the antibody suggesting the likelihood of complex regulatory relationships between these proteins. The percentage of m6A_IP/Input mRNA of GFP, SON, and these genes ranged from 2-6% (Figure 3B). The levels of their mRNA (except that of DF2) were increased in cells transfected with siDF2 and enriched in the IP fraction, compared with the Input (Figure S4B). These have been identified as m6A containing transcripts in several m6A mapping studies (Linder et al., 2015, Chen et al., 2019). No significant enrichment of these genes was observed in the control IP/Input (%) using an anti-gapdh antibody (Figure S4C). The ribosomal protein L30 (RPL30) and gapdh mRNA, on the other hand, were not enriched and therefore not subjected to m6A methylation.

To identify regions containing an m6A-modification on the GFP transcript, fragmented, enriched mRNA from cells treated with siNC or siMETTL3 - an m6A writer - was immunoprecipitated with an anti-m6A antibody and subjected to RT-PCR. Depleting METTL3 would be expected to reduce the levels of m6A methylation in substrate mRNAs compared to control cells. We identified two regions, the first between nt1-181 at the start of the transcript (GFP1) and the other flanking the stop codon (GFP806) (Figure 3C). The level of GFP transcript in these two regions was enriched in the m6A_IP (Figure S4D). The presence of two “consensus” m6A sites (GGAC) in the GFP806 region near the stop codon conforms to what has been found in other mammalian cells. It should be noted that the presence of the m6A site in the GFP1 region is not conclusive as the anti-m6A antibody used is known to also bind m6Am which is the first nucleotide after the 7- methylguanosine cap of a certain mRNAs (Linder et al., 2015).

3.5 Regulation of transgene mRNA GFP by ALKBH3 is m1A mediated

As the cell-based assays indicated the potential presence of m1A on the GFP transcript, we then performed immunoprecipitation with an anti-m1A antibody using fragmented, enriched mRNA from cells transfected with either siNC or siALKBH3 and the GFP expression vector. We found that the

well-characterized m1A site at nucleotide 1322 in human 28S rRNA, which is known to be conserved between mouse and human, is also conserved in CHO cells and located at nucleotide position 1146 (Genbank accession number NR_045212). The m1A_IP/Input ratio of 28S rRNA, which is known to be highly m1A methylated (Safra et al., 2017; Li et al., 2017) was 13-27%, compared to that of the m1A negative region 28S_304 (0.26% to 0.37%) (Figure 4A). We identified two regions in GFP, one of which (91 nucleotides) is located at the beginning of the transcript (GFP1S) and a second between nucleotides 659 and 835 (GFP659), both with an m1A_IP/Input ratio at least 7-fold higher than that of the "negative" region GFP1017 (0.33% to 0.76% versus 0.05% to 0.08%) (Figure 4B, 4C). However, only the GFP659 region showed the delayed reverse transcription (Figure S5C) associated with the presence of an m1A modification, similar to that of the 28S rRNA (Figure S5A), suggesting that the immunoprecipitation of the G1S region could be due to non-specific binding by the anti-m1A antibody (Grozhik et al., 2019). The levels of GFP were consistent with those of m1A_IP/Input (%) data (Figure S5D). The GFP659 region contains a GA rich motif, identified as m1A sites from mapping studies using two different approaches; an antibody-based and an antibody-independent approach (Yi et al., 2016; Zhou et al., 2019).

The level of GFP determined by RTPCR was lower in the Input fraction of cells treated with siALKBH3 (Figure S5D) suggesting that ALKBH3 affects GFP at the mRNA level, whereas an increase was observed in the transcript level of 28S_rRNA (Figure S5B) suggesting the underlying mechanism of m1A methylation of rRNA and mRNA are unlikely to be the same.

3.6 Expression of EPO in CHO cells is subjected to epitranscriptomic regulation by the m6A regulators

Having established that a model transgene, GFP, despite containing no predicted m6A sites, was regulated by the m6A machinery in CHO cells, we were interested in determining whether the production of a more commercially relevant therapeutic protein, EPO, would also be impacted by this mechanism and whether it could be used as a strategy to improve protein yield. The EPO transcript contains two predicted m6A sites of moderate score near the stop codon (Figure 5A). Given the positive impact of targeting the YTHDF2 protein in cells expressing GFP, we took the same approach in CHO-K1 cells transiently transfected with an EPO expression vector. Depletion of the m6A reader

resulted in an increase in yield of EPO by more than 200% (Figure 5B). On the other hand, depletion of either DF1 or 3 resulted in reduced EPO titre. It was also interesting to note that knocking down the levels of any of the YTHDF proteins had a small negative effect on cell viability (less than 10%) and a more substantial effect on cell density (~20%). However, this also indicates that the improvement in yield is underpinned by a significant increase (>2-fold) in specific cellular productivity.

4 Conclusions

Research in the last few years has highlighted the variable nature of the prevalent m6A modification and its impact on diverse biological processes. A few recent studies have implicated that the m1A mark is also a dynamic modification responsive to changing physiological and environmental conditions such as mammalian cells are exposed to in a bioreactor. These modifications eventually affect mRNA fate and translation efficiency-potentially impacting the production of biopharmaceutical proteins from cells. This study provides the first evidence of the significance of the m6A readers DF1-3 on the expression of a model transgene, GFP which is predicted not to contain any m6A sites. In particular, silencing of DF2, the main m6A reader that regulates mRNA stability has a pronounced impact on protein expression in CHO cells. A similar impact of DF2 knockdown was also observed on the expression of human EPO, a drug used for the treatment of some forms of anemia. This study is also the first to show that the expression of an mRNA can also be subjected to co-ordinated modulation of both m6A and m1A regulators. We also present the first genetic data implying that the adenosine nucleotides targeted for m1A methylation are possibly read by an m6A reader protein, as suggested by the recent data (Seo & Kleiner, 2020; Zheng et al., 2020) however, this requires further evidence.

In summary, we identified DF2 as a key player in the regulation of transgene expression in CHO cells. Knockout of DF2 positively affected the level of both a reporter GFP and the therapeutic protein EPO. In particular, these insights open the prospect of new epi-transcriptomic-based approaches to mammalian cell line engineering for recombinant protein production. These approaches could entail engineering the expression of various host cell readers, writers and erasers of these methyl groups on a recombinant transgene, as described here, or another strategy might include the placement of

specific methylation motifs within the transgenic expression cassette itself in order to influence the fate of the transcript, e.g. increased stability or translation. It will also be important to understand the wider influence of epitranscriptomic engineering of producer cell lines on other important elements of recombinant protein production, in particular critical product quality attributes such as glycosylation for example. This study demonstrated that while YTHDF2 depletion improved cell-specific productivity, there was also an impact on cell growth/density. Ongoing transcriptomic studies will help reveal the more widespread effects of this novel engineering strategy on various cellular pathways.

[Supplementary material is available at Biotechnology Journal online.]

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FIGURES LEGENDS

Figure 1. Impact of m6A and m1A regulators on GFP expression

Expression of GFP in CHO cells transfected with siRNAs for m6A writers and readers (**A**), m6A and m1A erasers (**D**) are reported as normalized Mean Fluorescence Intensity (MFI); values are the means ± standard deviation (SD), n = 3. Relative expression (RQ) of m6A, m1A regulators and GFP (**B, C, E**) determined by qRTPCR. siRNA for negative control (si_NC), METTL14 (M14). RQ of METTL14 and WTAP in cells transfected with siRNA for both METTL14 and WTAP (M14&WTAP_M14, M14&WTAP_WTAP). RQ of SON gene used as a methylated transcript control in cells transfected with siRNA for METTL13 (METTL3_SON). p <=0.05 (*); p <=0.01 (**).

Figure 2. Impact of knockdown of erasers and over-expression of DF2 on GFP expression

Relative expression (RQ) of eraser determined by qRTPCR (**A**). Over-expression of DF2 (CMV-DF2) determined by Western blot analysis using an anti-FLAG antibody (**B**). Impact of knockdown of erasers and over-expression of DF2 on GFP expression, shown as MFI (**C**) and RQ (**D**). Values are the means ± SD, n=2.; blue arrow on the right is the expected molecular weight (65KDa) of DF2 protein and 2 FLAG epitopes; ALKBH3 (BH3), ALKBH5 (BH5). p <=0.05 (*); p <=0.001 (***).

Figure 3: GFP mRNA is immunoprecipitated by an anti-m6A antibody

RQ of GFP in m6A_IP and Input fractions (**A**). Percentage of m6A_IP/Input mRNA of GFP and selected genes from cells co-transfected with siRNA (NC or DF2) and GFP, immunoprecipitated using an anti-m6A antibody (**B**). Percentage of m6A_IP/Input of different regions of GFP mRNA from cells co-transfected with siRNA (NC or METTL3) and GFP immunoprecipitated using an anti-m6A antibody (**C**). Values represent the mean ± SD (n=2). Immunoprecipitate (IP).

Figure 4: GFP mRNA is immunoprecipitated by an anti-m1A- antibody

Conserved m1A site on CHO 28S rRNA (GenBank accession number NR_045212) (red letters) and regions used in qRTPCR (light blue lines) are shown (**A**, top); Percentage of m1A_IP/Input of 28S rRNA (**A**, bottom). Potential m6A (GACT, blue vertical lines) and potential m1A sites (red letters) in GFP and the overlapping regions amplified by qRTPCR (light blue lines) are shown (**B**); Percentage of m1A_IP/Input of different parts of GFP mRNA from cells co-transfected with siRNA (NC or

ALKBH3) and GFP immunoprecipitated using an anti-m1A antibody (**C**). Values represent the mean ± SD (n=2). Immunoprecipitate (IP).

Figure 5: Impact of knockdown DF1-3 on EPO expression

Predicted m6A sites on EPO mRNA (**A**); Western blot analysis of EPO expression in CHO-K1 cells co-transfected with siRNA using an anti-human EPO antibody and quantification (n = 2) (**B**). Molecular weight (M), Standard (SD) EPO in ng (1, 52). Blue arrow on the left is the expected molecular weight (38kD) of glycosylated EPO. Impact of knock-down m6A readers DF1-3 on cell viability and growth (**C**). p <=0.01 (**), p<= 0.001 (***).