Use of the MS2 aptamer and coat protein for RNA localization in yeast: A response to “MS2 coat proteins bound to yeast mRNAs block 5′ to 3′ degradation and trap mRNA decay products: implications for the localization of mRNAs by MS2-MCP system”

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ABSTRACT

The MS2 system has been extensively used to visualize single mRNA molecules in live cells and follow their localization and behavior. In their Letter to the Editor recently published, Garcia and Parker suggest that use of the MS2 system may yield erroneous mRNA localization results due to the accumulation of 3′ decay products. Here we cite published works and provide new data which demonstrate that this is not a phenomenon general to endogenously expressed MS2-tagged transcripts, and that some of the results obtained in their study could have arisen from artifacts of gene expression.

Keywords: MS2 aptamer; MS2 coat protein; mRNA localization; mRNA decay; P-bodies

In the recent letter by Garcia and Parker (2015), the authors raise an important issue—e.g., whether the MS2 coat protein (MCP) affects the stability of an MS2 aptamer-tagged transcript (i.e., containing multiple MS2 stem-loops) upon coexpression in yeast cells. Accordingly, this could stabilize downstream elements (e.g., 3′UTR sequences), prevent their decay, and thereby give a false interpretation of the localization of the full-length transcript. Since many groups have used the MS2 system to label and localize transcripts for over 15 years, cumulating in numerous papers, their claim calls into question the validity of these studies.

The authors’ interpretation of the results, which is based entirely on Northern blot analyses, is that many, if not most (e.g., >70%) of the transcripts visualized by this system in yeast may actually be 3′ decay fragments that accumulate due to inhibition of the cytoplasmic 5′ to 3′ exonuclease, Xrn1. Although we do not dispute the fact that such fragments might accumulate under certain conditions (as we also demonstrate below), we note that there are several problems with their interpretation that raise questions about the conclusions of their letter. These are detailed below.

First, no actual mRNA imaging data are provided in the letter to support their claim. Of note, 3′ RNA fragments resulting from Xrn1 inhibition [by a poly-G (pG) tract] were previously imaged and shown to colocalize within P-bodies (PB) (Sheth and Parker 2003). However, the fragments described in Garcia and Parker (2015) either may or may not be similar in their behavior (e.g., accumulating in PBs). Indeed, MS2-labeled *MFA2* mRNA was not shown to colocalize with PBs in wild-type (WT) cells (Sheth and Parker 2003). It is possible that these fragments accumulate in a unique location (e.g., the nucleus) or result from an accumulation in only a subpopulation of cells in the yeast culture. However, such information cannot be derived from Northern blots. Thus, the question of whether the 3′ decay products act as pseudo-full-length transcripts in imaging experiments, thereby affecting the interpretation of mRNA localization data, is never actually addressed.

Second, both the data as presented in the figures (listed herein as Figure X [G&P 2015]) and the experimental design raise questions about the relevance or interpretation of some

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of their results. In brief: (i) No minus MCP controls are shown in Figure 2A–C (G&P 2015). Thus, it is difficult to determine whether MCP expression indeed elicits an Xrn1-dependent phenotype for these mRNAs, as suggested by the authors. Alternatively, the accumulation could result from the RNA aptamer itself inhibiting Xrn1 (which is an important issue, but raises other questions) or for other reasons unknown; (ii) the authors suggest that the accumulation of mRNA decay fragments in post-diauxic shift (PDS) cells depends upon the presence of Xrn1 (Fig. 1 [G&P 2015]). However, Xrn1 protein was reported to segregate apart from PB-associated decay factors into eisosomes (a unique compartment of the plasma membrane) under conditions of PDS (Grousl et al. 2015). Thus, Xrn1 might not be available to participate in mRNA decay during PDS (perhaps mimicking the situation found in *xrn1Δ* cells). Thus, the 3′ fragments detected in PDS cells may have accumulated during log-phase growth. However, during log-phase growth (Fig. 1D [G&P 2015]), the *xrn1Δ* lane shows a fragment smear similar to *XRN1* lanes, so the involvement of Xrn1 is unclear (see also point vi); (iii) most of the results are derived from plasmid-based overexpression (rather than endogenous gene expression), which could yield false findings due to the segregation of overexpressed mRNAs into PBs (discussed below). In Figure 2C (G&P 2015), the sole experiment where an endogenously expressed mRNA (*PGK1*; *YCR012W*) is analyzed, the signal strength for full-length MS2-tagged *PGK1* mRNA is several-fold weaker than the untagged mRNA, suggesting that the tagged gene is either transcribed at a lower rate or that the tagged MCP-bound mRNA is unstable (or both). Thus, the origin of the 3′ fragments may

relate to the insertion of the MS2 aptamer rather than the proposed inhibition of Xrn1 by MCP and, therefore, the source of the fragments could be transcription rather than decay; (iv) the authors expressed *ASH1* from a *GAL1* promoter, which is known to alter its normal mechanism of localization compared to *ASH1* expressed from its own promoter (Fundakowski et al. 2012). Therefore, the fragments in this case can result from either cleavage or degradation of the mislocalized mRNA; (v) the authors did not measure transcription and decay rates in the wild-type cells versus *xrn1Δ* cells. Since the lack of Xrn1 reduces transcription (e.g., two- to fivefold, as shown by us in Table 1, for the same genes examined in their letter) and impairs transcription elongation (Haimovich et al. 2013; Medina et al. 2014), it could give a false impression that there is an apparent reduction in the amount of decay fragments; and (vi) finally, the authors quantify some lanes of their Northern blots (Table 1 [G&P 2015]), but do not show quantitative data for the control lanes (i.e., samples lacking GFP-tagged MCP expression or lacking Xrn1 [*xrn1Δ*]). Therefore, the reader can only compare between lanes in a qualitative manner.

Third, the letter by Garcia and Parker (2015) does not cite numerous publications in which the MS2-MCP system was used to image these and other mRNAs. In particular, this includes studies in which the issue of transcript stability was directly examined. Indeed, several publications have shown that the transcripts detected by the MS2-MCP are intact and that their copy number and localization is similar to untagged endogenously expressed mRNAs. Examples include (i) comparable mRNA localization of either *ASH1* mRNA

TABLE 1. Fold-change in transcription, RNA abundance, stability and 3′ end reads

Name	Systematic name	Fold-change <i>xrn1Δ</i> /WT ^a			RS group ^{a,d}	RaPID 3′/5′ ratio ^e
		TR ^b	RA ^c	RS ^d		
<i>ASH1</i> ^f	YKL185W	0.32	0.73	2.42	Moderate	1.70
<i>PGK1</i> ^f	YCR012W	0.18	0.29	1.71	Moderate	ND ^g
<i>MFA2</i> ^f	YNL145W	0.54	1.47	2.54	Moderate	ND
<i>QCR8</i> ^f	YJL166W	0.37	0.78	2.00	Moderate	ND
<i>PEX14</i>	YGL153W	0.40	0.88	2.45	Moderate	0.45
<i>OXA1</i>	YER154W	0.55	0.80	1.40	No change	0.43
<i>OM45</i>	YIL136W	0.68	0.60	0.75	No change	1.82
<i>ATG8</i>	YBL078C	0.52	1.00	1.99	Moderate	0.73
<i>ABP1</i>	YCR088W	0.34	0.45	1.70	Moderate	3.80
<i>MYO4</i>	YAL029C	0.29	1.44	5.37	High	3.50
<i>EXO70</i>	YJL085W	0.39	0.69	1.84	Moderate	2.70
<i>SRO7</i>	YPR032W	0.44	1.01	2.32	Moderate	1.00
<i>MYO2</i>	YOR326W	ND	ND	ND	ND	3.06

^aData taken from Haimovich et al. (2013).

^b(TR) Transcription rate ratio, as determined by genomic run-on assay.

^c(RA) RNA abundance ratio, as determined by cDNA hybridization on in-house prepared nylon membrane macroarrays.

^d(RS) RNA stability ratio, as determined by thiolutin shutoff assay. RS groups were arbitrarily divided based on an increase in the RS ratio, as follows: “no-change” (<1.5-fold), “moderate” (between 1.5 to threefold) and “high” (greater than threefold).

^e(RaPID 3′/5′ ratio) Ratio of highest RNA-seq peak downstream from MS2 aptamer repeat sequence to highest peak upstream of the MS2 aptamer repeat sequence, as determined by RaPID-RNA-seq (see Fig. 1 and main text for details).

^fThese mRNAs were examined by Garcia and Parker (2015).

^g(ND) Not determined.

(Long et al. 1997; Bertrand et al. 1998; Gu et al. 2004; Andoh et al. 2006) or *ABP140* mRNA (Kilchert and Spang 2011) localization using different methods of detection (e.g., FISH and the MS2 and U1A aptamer systems); (ii) the copy number of an MCP-labeled *SUC2* transcript does not outnumber single-molecule FISH labeling of the *SUC2* ORF (Kraut-Cohen et al. 2013), indicating that there is no excess of 3' mRNA fragments; (iii) the copy number of *MDN1* mRNA tagged with either the MS2 or PP7 aptamer in cells either expressing or lacking the appropriate coat protein is similar to endogenous *MDN1* levels (Hocine et al. 2013); and (iv) MS2-MCP detection of the localization of mRNAs encoding peroxisomal proteins was validated by RT-PCR analysis of mRNAs associated with isolated peroxisomes (Zipor et al. 2009).

Despite the abovementioned issues, which initially led us to question their conclusions, we performed several additional analyses in order to test their hypothesis and further investigate this important issue. First, we quantitatively measured whether the MS2-MCP system affects the cellular ratio of mRNA fragments (e.g., either 3' or 5' to the MS2 aptamer repeat sequence). We analyzed RNA-seq data from mRNA pulldown (RaPID [Slobodin and Gerst 2010, 2011]) experiments, in which we affinity-purified MS2-tagged mRNAs using MCP-GFP fused to streptavidin-binding peptide (MCP-GFP-SBP) and immobilized streptavidin. RaPID employs formaldehyde to chemically crosslink RNA and protein, and is performed under conditions that maintain RNA integrity (e.g., prechilled buffers to prevent PB formation, added RNase inhibitors, etc.). For some mRNAs (e.g., *ASH1*, *OM45*, *ABP1*, *EXO70*, *MYO2*, and *MYO4*) we did observe a 1.7–3.8-fold increase in 3'UTR reads, compared to the ORF reads (Table 1; Fig. 1A–C). In contrast, we observed no difference or a mild decrease in the number of reads along the length of some MS2-tagged transcripts (e.g., *ATG8* and *SRO7*, Table 1; Fig. 1D) or even a twofold decrease in reads in the 3'UTR of other mRNAs (e.g., *PEX14* and *OXA1*, Table 1; Fig. 1E,F). Thus, while the MS2-MCP system might cause an accumulation of reads toward the 3' end of genes, we detected it in only six of 10 cases. Furthermore, these increases could be artifacts of the affinity purification of 3' end-tagged mRNAs, since the sequencing of oligo(dT)-purified untagged mRNAs in yeast showed an enrichment in the reads of the 3' ends of mRNAs (Nagalakshmi et al. 2008). We also did not find any obvious correlation between an increase in the 3'UTR reads and the effect that the lack of Xrn1 has on the transcription, abundance, and/or stability of these mRNAs (Table 1).

As described above, most of the experiments performed by Garcia and Parker (2015) were performed using multicopy plasmid-based gene overexpression. In RaPID-mass spectrometry studies performed in our laboratory, we noted that known PB proteins coprecipitate with MS2-tagged mRNAs that are overexpressed from plasmids (e.g., Xrn1, Sbp1; data not shown). In contrast, PB proteins were never

observed to coprecipitate with MS2-tagged mRNAs expressed from the genome under their native promoter. As a test case, we examined the PB-association of MS2-tagged *MFA2* mRNA, since we suspected that the 3' fragments are likely to accumulate in PBs. Furthermore, either U1A($\times 16$) or MS2($\times 2$)-tagged *MFA2* mRNA was recently suggested to localize to PBs and to affect mating in yeast upon its overexpression from a centromeric plasmid (for U1A) or multicopy plasmid (for MS2) under a constitutive *GPD* promoter (in both cases) (Aronov et al. 2015). Since these represent an extreme case of mRNA overexpression, we compared three strains with differing levels of *MFA2* expression: (i) MS2($\times 12$)-tagged *MFA2* mRNA expressed from the genome under its native promoter (named herein as *MFA2*-MS2); (ii) MS2($\times 12$)-tagged *MFA2* expression from the genome under a galactose-inducible (*GAL1*) promoter (named herein as *gGAL1*-*MFA2*-MS2); and (iii) MS2($\times 12$)-tagged *GAL1*-*MFA2* expressed from a multicopy plasmid (named herein as *pGAL1*-*MFA2*-MS2). First, we noticed that under galactose-inducing conditions, the generation time of strain *pGAL1*-*MFA2*-MS2 was 4 h, compared to a generation time of ~ 2.5 h of the other strains. This indicates that cells overexpressing *MFA2* from plasmids are cell cycle-inhibited, which may result from the stress of gene overexpression. Expression levels of *MFA2*-MS2 mRNA were measured as the number of mRNA granules per cell and the number of mRNA transcripts per granule. The results show the expected differences in mRNA levels given the different expression conditions (Fig. 2A,B). Next, we found that the *pGAL1*-*MFA2*-MS2 strain formed more PBs when compared to the other two strains (Fig. 2C,D) and most of these PBs appeared larger than those of the other strains (not shown). Furthermore, multicopy expression of *MFA2*-MS2 (*pGAL1*-*MFA2*-MS2) resulted in an increased colocalization of the mRNA with PBs compared to native expression (*MFA2*-MS2) or mild overexpression conditions (*gGAL1*-*MFA2*-MS2) (Fig. 2E–G). We note that large mRNA granules were more likely to colocalize with PBs when compared to small mRNA granules (Fig. 2G). Thus, high levels of mRNA expression can result in cellular stress and mRNA mislocalization to PBs, which may potentially affect the mRNA degradation process. Interestingly, upon mild amino acid starvation (e.g., 25% of normal levels), even a low level of *MFA2* overexpression (*gGAL1*-*MFA2*-MS2) resulted in increased PB formation and increased mRNA/PB colocalization when compared to native expression (*MFA2*-MS2; Table 2). Therefore, mRNA overexpression may stress the cells into producing PBs depending upon the growth conditions. These PBs likely segregate the extra transcripts (or transcript fragments) from the translation machinery and possibly destine them for degradation.

Since the above experiments (Fig. 2) did not discriminate between full-length MS2-tagged mRNAs and 3' fragments, we extracted RNA from the above strains and compared the ratio of ORFs to the 3'UTR amplicons by RT-qPCR. Consistent with the results of Garcia and Parker (2015), the

FIGURE 1. Affinity purification of MS2-tagged mRNAs using MS2 coat proteins reveals that 3' end fragments accumulate only in some, but not all, mRNAs. Yeast genes encoding the indicated mRNAs were MS2-tagged using m-TAG (Haim et al. 2007). Yeast cells were cultured in synthetic complete (SC) media, and expression of MCP-GFP-SBP was induced by culturing the cells in SC-methionine for 1 h. RaPID was performed as previously described (Slobodin and Gerst 2010, 2011). Libraries were constructed for RNA-seq from the affinity-purified RNA, as described for the control (non-strand-specific) library in Levin et al. (2010), except that the oligo(dT) selection and RNA fragmentation procedures were omitted. In addition, only random hexamers were used for cDNA synthesis and Ampure beads were used for size selection. Libraries were sequenced using an Illumina HiSeq2000 sequencer (paired-end 76 base reads) to a depth of $\sim 10^7$ reads. RNA-seq reads were aligned to the *S. cerevisiae* reference genome sequence using the TopHat2 program (Kim et al. 2013) (v.2.0.10). Read base-level coverage across the genome was computed using the "SAMtools depth" function and plotted for each corresponding gene. Results are shown for six representative mRNAs. X-axis shows the nucleotide position along the indicated chromosome. The graph shows the number of RNA-seq reads along the mRNA sequence. Blue line on the x-axis indicates the length of the ORF. Purple arrow indicates the insertion position of the MS2 aptamer repeats (12; MS2-12 $\times$ SL). See Table 1 for the ratio of the 3' end/ORF highest peaks.

strains that overexpressed *MFA2*-MS2 mRNA (e.g., *gGAL1-MFA2*-MS2 and *pGAL1-MFA2*-MS2) showed a significant enrichment of the 3' end compared to the ORF amplicon (Fig. 3). Unlike the results of Garcia and Parker, however, we found that expression of the MCP-GFP($\times 3$) had no effect upon this ratio. Thus, although the mRNA granules might possibly contain 3'UTR decay fragments, Garcia and Parker's main claim that binding of MCP-GFP to the MS2-tagged *MFA2* mRNA is the cause of decay fragment accumulation (due to inhibition of Xrn1) is incorrect and would necessitate a more direct proof (e.g., by performing *in vitro* mRNA degradation assays). Interestingly, we found that endogenously expressed *MFA2*-MS2 gave a slight increase in the 3'UTR/ORF amplicon ratio in comparison to untagged

endogenous *MFA2* (Fig. 3). This indicates that insertion of the MS2($\times 12$) aptamer repeat sequence might affect degradation of the tagged mRNA. This may occur in the small subpopulation of cells that contain granules bearing a high number of mRNAs per granule (e.g., only 13% of granules contain >3 mRNAs/granule) (Fig. 2A,B, *MFA2*-MS2) and/or in cells in which the mRNA granule colocalizes with a PB ($\sim 3\%$ of cells) (Table 2; Fig. 2E,F, *MFA2*-MS2). Further analysis is required to pinpoint the cause of the accumulation of 3'UTR fragments detected by RT-qPCR and the RaPID-RNA-seq experiments and is beyond the scope of this letter.

All told, the evidence we present here strongly suggests that the use of plasmid-based overexpression systems to localize mRNAs and Northern blot analysis to detect mRNA decay

FIGURE 2. Association of MCP-GFP(×3)-labeled *MFA2*-MS2 mRNA granules with P-bodies increases with mRNA expression levels. The tagging of *MFA2* with the MS2 aptamer repeats (×12) was done using m-TAG in yeast strain BY4741 (EUROSCARF), as previously described (Haim et al. 2007). The native genomic promoter of *MFA2* was replaced by homologous recombination with the *GAL1* promoter using plasmid pFA6a-KanMX6-PGAL1 (EUROSCARF) to yield *GAL1*-*MFA2*-MS2. Proper integration of the MS2 aptamer repeats and the *GAL1* promoter were verified by colony PCR analysis. To create a multicopy plasmid of *GAL1*-*MFA2*-MS2, the whole-genomic fragment of *GAL1*-*MFA2*-MS2 was PCR amplified and inserted into plasmid pRS423. To visualize PBs, we expressed Dcp2-RFP from a plasmid (pRP1152, a gift from R. Parker). To visualize mRNA granules, we expressed MCP-GFP (×3) from a plasmid (pUG36-CP-GFP×3) under the *MET25* promoter (Haim et al. 2007). However, to avoid amino acid starvation (which can potentially increase PB formation), we relied on the “leakiness” of the *MET25* promoter to provide enough MCP-GFP(×3) protein expression for mRNA granule visualization (Hocine et al. 2013). It should be noted that only ~40% of *MFA2*-MS2, ~50% of *gGAL1*-*MFA2*-MS2, and 75% of *pGAL1*-*MFA2*-MS2 cells expressed visible levels of MCP-GFP(×3), while >90% of the cells expressed visible levels of Dcp2-RFP. Cells were propagated in synthetic complete media lacking uracil/leucine or uracil/leucine/histidine (selectable markers of the plasmids) and containing 3.5% galactose. An overnight culture was diluted and cells were allowed to divide for at least seven generations prior to imaging. When the cultures were at $0.5\text{--}1 \times 10^7$ cells/mL, a small sample was plated on concanavalin-A coated glass bottom dishes (P35G-1.5-14-C MatTek) and cells were imaged using a Delta Vision (DV) system with an Olympus IX-71 microscope equipped with a mercury lamp (Olympus), PlanApo 100× 1.35 NA oil immersion objective (Olympus), and a CoolSNAP HQ CCD camera (Photometrics). Z-stack imaging (0.6 μm steps; a total of five Z-sections each) was performed with an automated stage (Applied Precision) using DV software, at 0.0645 μm/pixel. Exposure time for GFP was 80 msec (total 0.95 sec) and for RFP, 300 msec (total ~3 sec). We imaged ~400 cells in 50 different fields for each strain. (A) Histogram depicting the distribution of the number of mRNA granules/cell for each strain. (B) Graph depicting the estimated number of *MFA2* mRNA transcripts per mRNA granule, as calculated using the transcription-site analysis tool of the FISH-quant program (Mueller et al. 2013). Each symbol on the graph represents a single mRNA granule. (C) Histogram depicting the distribution of the number of PBs/cell for each strain. (D) Table summarizing the average number of mRNA granules, mRNA transcripts, and PBs per cell for each strain. (E) Graph depicting the percent colocalization of mRNA granules with PBs. Only cells with both GFP and RFP signals were considered for this analysis. (F) Graph depicting the percent PB colocalization with mRNA granules. Only cells with both GFP and RFP signals were considered for this analysis. Note: For E and F in cases where the mRNA and PBs were adjacent (i.e., 1–3 pixels apart), the mRNA and PB were considered to be colocalized. This is because mRNA–PB separation might result from movement of the granules/PBs during the long imaging duration. This was observed primarily with small granules/PBs. (G) Images of *pGAL1*-*MFA2*-MS2 cells showing large mRNA granules colocalizing with PBs (arrowheads in Merge) and a few small granules [arrows in MCP-GFP(×3)]; each containing at least one mRNA with no visible PB colocalization.

TABLE 2. Association of MFA2-MS2 mRNA granules (detected using MCP-GFP) with P-bodies upon mild amino acid starvation

Strain	Average # of mRNA granules/cell	Average # of PBs/cell	Colocalization (%)	
			mRNA with PBs	PBs with mRNA
<i>MFA2-MS2</i>	0.35	0.09	7.7%	19.2%
<i>gGAL1-MFA2-MS2</i>	1.09	0.24	27.3%	80.0%

fragments generated under those conditions can give a false impression of global mRNA protection (Garcia and Parker 2015) and, in some cases, probably altered mRNA localization (Fundakowski et al. 2012; Aronov et al. 2015). Although our RaPID-RNA-seq and RT-qPCR data suggest that there may be accumulation of 3' end fragments in some cases, this is not a general rule and needs to be examined on an individual case-by-case basis. This is important to determine if the accumulation of 3'UTR fragments is not an artifact of gene overexpression, is limited to a subpopulation of cells, or is due to other causes. For instance, it is possible that under some conditions, the MS2 aptamer creates a structure similar to that of the Xrn1-inhibiting RNA of flavivirus (Chapman et al. 2014) that is further stabilized by MCP. Alternatively, the exosome, which degrades mRNAs from 3' to 5', may play a role in the selective accumulation of 3'UTR fragments for some mRNAs, but not for others.

In conclusion, we confirmed Garcia and Parker's original observation that 3' fragments of MS2-labeled mRNAs may accumulate in yeast cells. However, we claim that the bulk of these fragments may be the result of overexpression and, at least for *MFA2-MS2* mRNA, it is not the result of MCP association with the mRNA. As opposed to Northern analysis, our imaging data suggest that these fragments could arise from a small, but visually distinct, subpopulation of cells that bear large granules that colocalize with PBs. Once a distinction can be made at the imaging level, one can concentrate on the majority normal cell population and, thus, eliminate scoring of the abnormal minority subpopulation that could result from the accumulation of 3' fragments. Therefore, we argue that the MS2 system can accurately predict mRNA localization in living cells when used properly (e.g., for endogenously expressed mRNAs or upon careful analysis of the localization/stability of the overexpressed mRNA). We therefore view the conclusions of Garcia and Parker (2015) as being incomplete.

NOTE ADDED IN PROOF

A commentary has been written in response to the submission of this letter (see Garcia and Parker 2016). Therein, the authors examined RNA extracted from yeast strains bearing the integrated MS2 aptamer sequences that are presented here in Figure 1, by Northern analysis. The authors find (Fig. 1 in Garcia and Parker 2016) that 3' fragments accumulate in these strains in the presence of MCP-GFP [MS2-CP-GFP(x3); Haim et al. 2007]. This matches our RNA-seq

data for MCP-precipitated transcripts in the cases of *ASH1*, *ABP1*, *EXO70*, *MYO2*, *MYO4*, and *OM45*, but not in the cases of *ATG8*, *PEX14*, and *OXA1*, where no accumulation of 3' reads was observed (Fig. 1). Garcia and Parker (2016) also confirm our hypothesis that the overexpression of MS2 aptamer-tagged mRNAs (e.g. *MFA2*) increases the amount of detectable 3' end fragments (Fig. 2 in Garcia and Parker 2016). Their Northern analysis suggests that 3' decay fragments derived from MS2 aptamer-tagged *MFA2* mRNA exist only in the presence of MCP-GFP (Fig. 2 in Garcia and Parker 2016), which is inconsistent with our RT-qPCR analysis (Fig. 3) that showed similar levels of fragments exist with or without MCP-GFP. The reason for this discrepancy is unclear, but could

FIGURE 3. RT-qPCR analysis of the ratio of *MFA2* mRNA 5' to 3' ends shows an increase upon gene overexpression. Strains *MFA2-MS2*, *gGAL1-MFA2-MS2*, *pGAL1-MFA2-MS2*, *MFA2-MS2::HIS3Δ3'UTR*, and a wild-type (WT; BY4741) control strain were transformed with plasmid pUG36-CP-GFPx3. Transformed and nontransformed strains were cultured in the same conditions as in Table 2. *MFA2-MS2::HIS3Δ3'UTR* was used as a negative control, since in this strain the *HIS3* cassette was not removed after insertion of m-TAG, thus, the 3'UTR of *MFA2* is not expected to be expressed. RNA was extracted from mid-log-phase cells ($OD_{600} = 0.4-0.6$) using MasterPure Yeast RNA Purification kit (Epicentre), and genomic DNA was extracted, as previously described (Harju et al. 2004). RNA samples were subjected to DNase treatment (RQ1 DNase, Promega) followed by reverse transcription using M-MLV Reverse Transcriptase (Promega). RT-qPCR was performed using SYBR Green Master Mix (ABI) on a LightCycler 480 Real Time PCR (Roche). The same pair of primers served in the amplification of cDNA and genomic DNA for each strain tested. Quantification cycle reads (Cq) obtained from using genomic DNA as the template served to normalize the Cq obtained for cDNAs and the $\Delta\Delta C_t$ algorithm was applied (Pfaffl 2001). Each of the tagged strains was normalized to the WT control. Depicted is the average $\pm$ SEM of $n = 4$ repetitive experiments.

result from differences in assay sensitivity, in which qPCR detects fragments that are undetectable by standard Northern analysis. This is important given the fact that the levels of full-length MS2-tagged MFA2 RNA are decreased in the absence of MCP-GFP($\times 3$) (Fig. 2 in Garcia and Parker 2016), which could reduce the detection of 3'UTR decay fragments by Northern analysis.

Thus, while we fully concur with Garcia and Parker (2015, 2016) that the presence of MCP or MCP-GFP can result in the accumulation of 3' decay products, we are not in agreement that Northern analysis is sufficient (by itself) to possibly disqualify mRNA localization data. In keeping with our cautionary message, we affirm that the localization of MS2-tagged messages should be examined on a case-by-case basis using endogenously expressed transcripts and in combination with RNA-seq, qPCR, or Northern analysis procedures to verify RNA integrity. In addition, it should be combined with quantitative analyses of mRNA granule size and/or colocalization with P-bodies to exclude suspicious subpopulations of granules from contributing to the interpretation of mRNA imaging results. Moreover, the use of colocalization procedures (e.g., co-FISH, as suggested by Garcia and Parker [2016]) or FISH-MS2 labeling (Kraut-Cohen et al. 2013) would go far to rule out the introduction of RNA localization artifacts.

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