



Friedrich Miescher Institute
for Biomedical Research

Master Thesis

Single-molecule imaging of mRNA to study translational regulation

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degree of Master of Science in Molecular Biology.*

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Abstract

will do that once we discussed

- stress granules hotly debated - one approach is to look at sg components individually - g3bp1 one of the core components - tethering seems to increase mrna stability - improved translation site imaging tool ready for usage

Keywords: SunTag Imaging, TREAT, mRNA Tethering, ?

Acknowledgments

Some thankyou note to Jeff, Daniel, Franka, Varun, and Susan.

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1

Introduction

1.1 mRNA Life Cycle

Transcription is the first step in gene expression in which a premature RNA molecule is made from a gene's DNA template. In eukaryotes, splicing, adding a 5' cap and poly-A-tail, as well as binding of cap- and RNA-binding proteins yields mature messenger RNAs (mRNAs). These mRNAs are then typically exported through nuclear pore complexes, which perforate the nuclear envelope. Once in the cytoplasm, the acquired protein coat determines the fate of the mRNA [1]. Translation, a process involving polysome assembly and protein coat remodeling, can occur immediately or delayed, such as in the case of various developmental transcripts. After translation is arrested, polysomes get disassembled and mRNAs are deadenylated. mRNAs can now be degraded or stored until internal or external signals call for their renewed translation. Subsets of these mRNAs can be packaged into RNA granules (Figure 1.1A). RNA granules are cytoplasmic, membrane-less aggregates containing various proteins involved in translation initiation and repression (reviewed in [2]).

Stress granules (SGs) are a prominent type of RNA granules that exclusively form under stress. Consensus suggests that, due to their enrichment in initiation factors, SGs exclusively contain translationally repressed mRNAs (reviewed in [3]). However, recent findings have reported otherwise [4]. While SG formation seems to be a conserved phenomenon throughout eukaryotes, their function remains unclear. SG formation was found to involve the nucle-

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ation through translationally silent messenger ribonucleoproteins (mRNPs). Proteins such as T-cell intracellular antigen-1 (TIA-1) [5] and Ras GTPase-Activating Protein-Binding Protein 1 (G3BP1) [6] have been found to induce

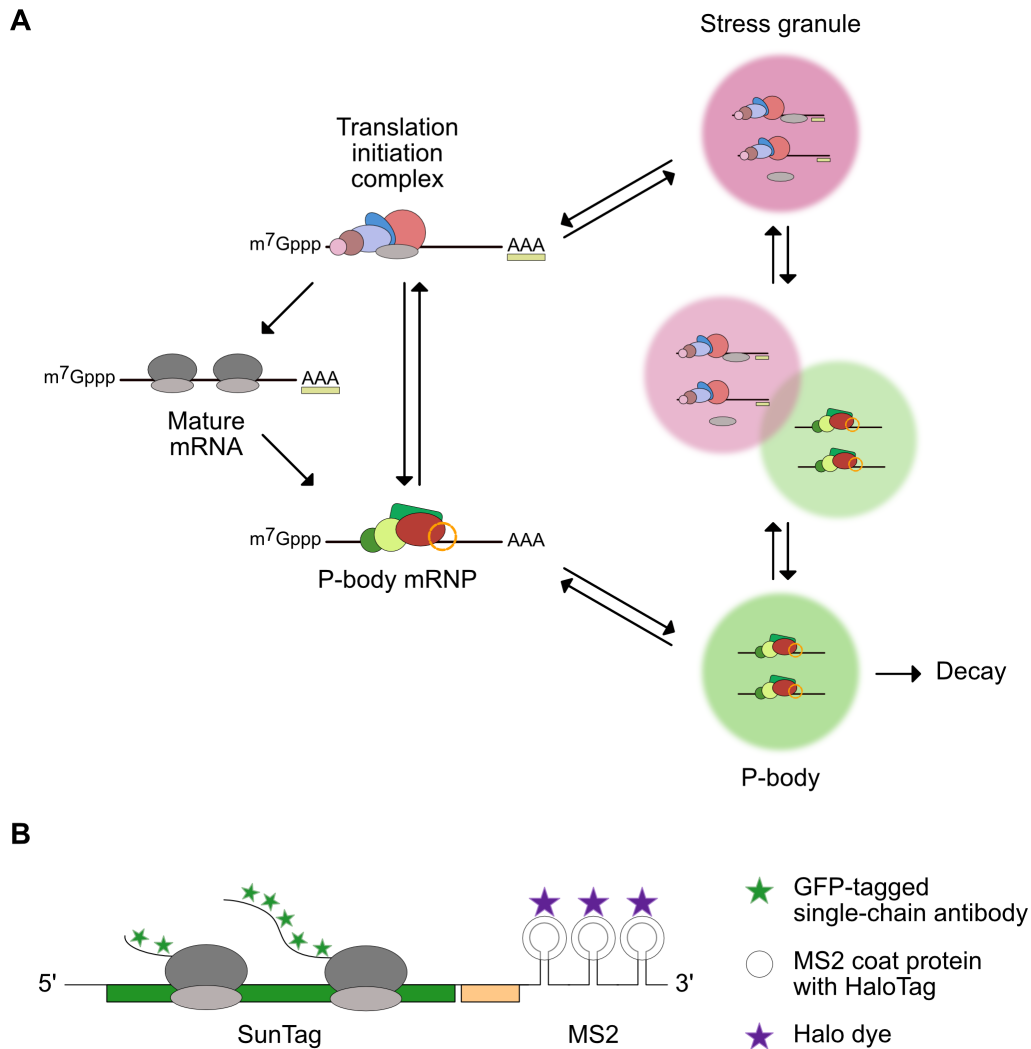


Figure 1.1: mRNA life cycle and translation site imaging. (A) Overview of a typical mRNAs life cycle ranging from translation to decay. (B) Schematic description of SunTag imaging to visualize and quantify translational activity on single-molecule level.

SG formation upon overexpression.

mRNA Processing Bodies (P-bodies) were originally described as mouse XRN1p (a highly conserved 5'-3' exonuclease) foci in the cytoplasm [7]. P-bodies, unlike stress granules, are present in unstressed physiology. However, stress can further increase their formation (reviewed in [8]). Beside co-translational mRNA decay through various RNases, P-bodies are considered one of the main sites of mRNA degradation.

1.2 Translation Site Imaging

To better understand the translational dynamics of single transcripts, a recent method has emerged. The stellar explosion supernova (SunTag) imaging approach allows for live-cell imaging-based analysis to visualize and quantify translation sites [9]. The SunTag system relies on three core components: a reporter RNA containing a SunTag cassette and MS2 stem-loops, a GFP-tagged single-chain antibody (scAB) against GCN4, and an MS2 coat protein fused to a HaloTag (Figure 1.1B). Currently available standard SunTag cassettes consist of 24 GCN4 repeats that, upon translation and exposure of the GCN4 epitopes, can be recognized and bound by the co-expressed scAB. As translation continues, more epitopes get revealed allowing more scABs to bind. This increases the local GFP concentration and thereby brightens the fluorescent signal. The MS2 coat protein-HaloTag fusion in conjunction with a soluble Halo dye is used to visualize the MS2 stem loops on the reporter RNA. Taken together, the system allows for accurate tracking of single RNAs and simultaneous brightness-inferred translational quantification.

2

Methods

2.1 Key Resources

Table 2.1: Antibodies

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| Alexa Fluor 647 | Abcam | ab150075 |
| TIA-1 | Abcam | ab40693 |

Table 2.2: Experimental Models: Cell lines

| Reagent or Resource | Source | Identifier |
|---|------------------------------|------------|
| Hela 11ht | Weidenfeld et al., 2009 [10] | N/A |
| Hela 11ht + scAB-GFP + Renilla-MS2 | This study | N/A |
| Hela 11ht + scAB-GFP + smGCN4-Renilla-MS2 | This study | N/A |
| Hela 11ht + scAB-GFP + SunTag-Renilla-MS2 | This study | N/A |

Table 2.3: Chemicals and Peptides

| Reagent or Resource | Source | Identifier |
|--------------------------------|-------------------------------------|-------------|
| Amino-11-ddUTP | Lumiprobe | 15040 |
| Atto 647N NHS ester | Sigma-Aldrich | AD 647N-31 |
| Bovine serum albumin | Sigma-Aldrich | A2153-50G |
| Dextran sulfate | Sigma | D6001-50G |
| Doxycycline | Sigma | D3891-1G |
| FluoroBrite DMEM | Life Technologies | A1896703 |
| Formamide (deionized) | Chemicon | S4117 |
| Ganciclovir | Sigma-Aldrich | G2536-100MG |
| JF585 HaloTag ligand | Grimm et al., 2015, 2017 [11, 12] | N/A |
| Lipofectamine 2000 | Invitrogen | 11668019 |
| Opti-MEM™ | Gibco | 31985070 |
| Paraformaldehyde (aqueous) | 20% Electron Microscopy Sciences | Sci-15713 |
| ProLong Gold Antifade Mountant | Molecular Probes, Life Technologies | P36935 |
| Puromycin | Invivogen | ant-pr-1 |
| Sodium Arsenite solution | Sigma | 35000-1L-R |

Table 2.4: Critical Commercial Assays

| Reagent or Resource | Source | Identifier |
|-------------------------------|--------|------------|
| <i>continues on next page</i> | | |

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| Reagent or Resource | Source | Identifier |
|---------------------------------|---------|------------|
| Bradford Protein Assay | Biorad | 5000006 |
| Renilla Luciferase Assay System | Promega | E2810 |

Table 2.5: Oligonucleotides

| Reagent or Resource | Source | Identifier |
|---|--------|------------|
| Oligodeoxyribonucleotides and primers are listed in Appendix A. | N/A | N/A |

Table 2.6: Recombinant DNA

| Reagent or Resource | Source | Identifier |
|--------------------------|-------------------------|-----------------|
| NLS-stdMCP-stdHalo | Voigt et al., 2017 [13] | Addgene #104999 |
| NLS-stdMCP-stdHalo-G3BP1 | This Study | N/A |
| NLS-stdMCP-stdHalo-Rh1 | This Study | N/A |
| pCAGGS-FLPe-IRESpuro | Beard et al., 2006 [14] | Addgene #20733 |
| Renilla-MS2v5 | This Study | N/A |
| scAB-GFP | Voigt et al., 2017 [13] | Addgene #104998 |
| smGCN4-Renilla-MS2v5 | This study | N/A |

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| Reagent or Resource | Source | Identifier |
|--------------------------|------------------------------|--------------------|
| SunTag-Renilla-MS2v5 | Wilbertz et al., 2019 [15] | Addgene #119945 |
| SunTag-Renilla-PP7-MS2v4 | Horvathova et al., 2017 [16] | N/A |

Table 2.7: Critical Equipment

| Reagent or Resource | Source | Identifier |
|--|----------------------|------------|
| 405 iBeam Smart | Toptica Photonics | N/A |
| 488 iBeam Smart | Toptica Photonics | N/A |
| 561 Cobolt Jive | Cobolt | N/A |
| 639 iBeam Smart | Toptica Photonics | N/A |
| 96-Well White Polystyrene Microplates | Costar | 07-200-589 |
| CFI Plan Apochromat Lambda 100x Oil/1.45 Objective | Nikon | N/A |
| CSU-W1 Confocal Scanner Unit | Yokogawa | N/A |
| Glass Coverslips | Paul Marienfeld GmbH | 117580 |
| iXon-Ultra-888 EMCCD Cameras | Andor | N/A |
| Mithras Multimode Microplate Reader LB 940 | Berthold | 38099 |

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| Reagent or Resource | Source | Identifier |
|---|------------------------------------|------------|
| MS-2000 Motorized Stage | Applied Scientific Instrumentation | N/A |
| TetraSpeck™ Fluorescent Microspheres Size Kit | Thermo Fisher Scientific | T14792 |
| Ti2-E Eclipse Inverted Microscope | Nikon | N/A |
| VS-Homogenizer | Visitron Systems GmbH | N/A |

Table 2.8: Software and Algorithms

| Reagent or Resource | Source | Identifier |
|-------------------------|------------------------------|--|
| Affinity Designer 1.8.2 | Serif (Europe) Ltd | affinity.serif.com/designer/ |
| Benchling 2020 | Benchling | benchling.com |
| Fiji 2.0.0-rc-69/1.52p | Schindelin et al., 2012 [17] | fiji.sc |
| Fluffy 0.2.2 | Eichenberger, 2020 [18] | github.com/BBQuercus/fluffy |
| KNIME 3.7.2 | Berthold et al., 2009 [19] | knime.com/knime-analytics-platform |
| PyMOL 2.3.3 | Schrodinger LLC. | pymol.org |
| Python 3.7.4 | Python Software Foundation | python.org |
| TrackMate v5.2.0 | Tinevez et al., 2017 [20] | imagej.net/TrackMate |

continues on next page

| Reagent or Resource | Source | Identifier |
|---------------------|-----------------------|---|
| VisiView 4.4.0 | Visitron Systems GmbH | visitron.de/ products/ visiviewr-software |

2.2 Method Details

Cell lines and culture details

The previously described HeLa-11ht cell line [10] was used for this study. The integrated Flp-RMCE (recombinase-mediated cassette exchange) site allows for controlled, single-copy, genomic integration of a target gene. In addition, to induce inserted target genes reversibly with doxycycline, cells are expressing a reverse tetracycline controlled transactivator (rtTA2S-M2). Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 4 mM L-Glutamine, and 10% v/v Fetal Bovine Serum (FBS) was used to culture HeLa cells. Cells were maintained at 37°C with 5% CO₂. Transient transfections were performed with Lipofectamine 2000 transfection reagent (Invitrogen) with Opti-MEM reduced serum medium (Gibco) according to manufacturer's instructions but scaled down to 0.5 µg plasmid DNA and 2 µl Lipofectamine 2000 per 1 ml growth medium.

Plasmid construction

To generate the G3BP1 coat protein fusion, the genomic sequence encoding the full-length G3BP1 Isoform 1 (Q13283-1) was inserted in-frame, downstream of the HaloTag sequence. A 33 nt linker sequence was kept between HaloTag and G3BP1 to ensure steric movement. Assembly was performed by PCR amplification of G3BP1 and the MCP-Halo-linker backbone, followed by Gibson cloning [21]. The resulting construct contains a constitutive UbiC promoter, SV40 NLS (nuclear localization signal), stdMCP (MS2 coat protein), HaloTag, 33 nt linker sequence, G3BP1 with a stop codon, and a Woodchuck

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Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) improving expression in lentiviral transfection (Figure B.1).

The GCN4 epitope containing the sequence for the smGCN4 reporter was synthesized into a pUC57-Kan expression vector. Assembly was performed by PCR amplification of smGCN4 and the *Renilla*-MS2v5 backbone followed by Gibson cloning. The resulting plasmid contains a Tet-CMV (cytomegalovirus) promoter followed by smGCN4, *Renilla* luciferase, FKBP domain with a stop codon, MS2v5 stem-loop cassette (24x), CTE (constitutive transport element, and SV40 polyA tail (Figure B.2).

Reporter cell line generation

A day before selection, HeLa cells were seeded into a 6-well plate. The targeting plasmid containing the reporter (2 µg) and pCAGGS-FLPe-IRESpuromycin plasmid (2 µg) were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturers' protocol [14]. On the next day, cells were preselected with 5 µl/ml puromycin (Invivogen). Two days later, puromycin was removed and replaced with fresh growth medium containing 50 µM ganciclovir (Sigma-Aldrich). The negative selection was performed for 10-14 days to get resistant colonies that had undergone RCME. Single-cell sorting into a 96-well plate was performed. Clones were tested for reporter expression using luciferase assays.

Renilla luciferase assays

Reporter production was measured with Promega's *Renilla* Luciferase Assay System. The described cell lines were seeded on 12-well plates. The next day, plasmid DNA was transfected once cells reached approximately 70% confluency as described above. On the subsequent day, the expression of reporters was induced with 1 µg/ml doxycycline (Sigma) for 3 hours unless otherwise specified. Stress was induced by adding 1 mM/ml sodium arsenite (Sigma) for the last 1 hour of induction. To measure recovery, cells were washed twice with

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PBS and replaced with fresh culturing medium. At the specified time points, cells were washed once with PBS and lysed with 250 μ l Passive Lysis Buffer per well. To ensure full cellular lysis, plates were gently shaken for 15 minutes at room temperature. 30 μ l lysate was transferred to 96-well EIA/RIA Plate (Costar) in triplicates. Bioluminescence was measured 2 seconds after injecting 12 μ l *Renilla* Luciferase Assay Reagent per well. Measurements were performed on the Mithras Multimode Microplate Reader LB 940 (Berthold). Data was normalized by protein concentration measured by standard Bradford Protein Assay. Means of at least 3 biological replicates (each with 3 described technical replicates) were calculated.

Single-molecule fluorescence in situ hybridization (FISH)

Single-molecule RNA detection against the *Renilla* coding sequence and MS2 (v5) was performed using Stellaris FISH probes (Biosearch Technologies). Probes targeting MS2 (v4) were made by enzymatic oligonucleotide labeling [22] with Amino-11-ddUTP (Lumiprobe) and Atto647-NHS (ATTO-TEC).

Cells were seeded on glass coverslips (Paul Marienfeld GmbH) placed in 12-well plates. The next day, cells were transfected as indicated. On the subsequent day, cells were treated as indicated, followed by two washes with PBS and fixation in 4% paraformaldehyde (Electron Microscopy Sciences) diluted in PBS for 5 minutes. Cells were washed thrice with PBS and permeabilized with 0.5% Triton X-100 diluted in PBS for 5 minutes (if immunofluorescence was performed) or overnight at 4°C in 70% Ethanol. Cells were washed two more times with PBS and prehybridized with wash buffer (2x SSC (Invitrogen), 10% v/v formamide (Abcam)) twice for 5 minutes. Coverslips were then incubated between 4 and 16 hours with hybridization solution (2x SSC, 10% v/v formamide, 10% v/v dextran sulfate, 0.5% v/v BSA, 200 nM FISH probes) at 37°C in humidified chambers. Cells were washed once more with wash buffer for 30 minutes and twice with PBS. Immunofluorescence staining was performed according to the “Immunofluorescence section from BSA blocking

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to mounting". Samples were then mounted on ProLong Gold Antifade Mountant with DAPI. Imaging was performed as described in the "Live-cell imaging" section using sequential, single-camera acquisition.

Immunofluorescence

Cells were seeded on glass coverslips (Paul Marienfeld GmbH) placed in 12-well plates. The next day, cells were transfected as indicated. On the subsequent day, cells were treated as indicated, followed by two washes with PBS and fixation in 4% v/v paraformaldehyde (Electron Microscopy Sciences) diluted in PBS for 5 minutes. Cells were washed thrice with PBS and permeabilized with 0.5% v/v Triton X-100 diluted in PBS for 5 minutes. Cells were washed two more times in PBS and incubated in 3% w/v bovine serum albumin (BSA) (Sigma-Aldrich) diluted in PBS for 1 hour. Primary antibodies (diluted in 1% w/v BSA in PBS) were incubated for 1 hour at room temperature or overnight at 4°C. After washing three times with PBS, the cells were incubated with secondary antibodies (diluted in 1% w/v BSA in PBS) for 1 hour at room temperature. Cells were washed twice with PBS and mounted on glass slides in ProLong Gold Antifade Mountant with DAPI. Imaging was performed as described in the "Live-cell imaging" section using sequential, single-camera acquisition.

Live-cell imaging

Cells were seeded on 35 mm glass-bottom μ -Dish (ibidi GmbH). The next day, cells were transfected as indicated. On the subsequent day, cells were treated as indicated. During the last 15 minutes of treatment, the medium was supplemented with JF585 HaloTag ligand, obtained from L. Lavis (Janelia Research Campus) [11, 12], at 100 nM final concentration. After incubation, cells were washed once with PBS and kept in FluoroBrite DMEM (Life Technologies) containing 10% v/v FBS and 4 mM L-glutamine. Cells were imaged within 15 minutes of medium exchange on an inverted Ti2-E Eclipse microscope (Nikon)

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with a CSU-W1 Confocal Scanner Unit (Yokogawa), two back-illuminated EM-CCD cameras iXon-Ultra-888 (Andor), an MS-2000 motorized stage (Applied Scientific Instrumentation), and VisiView imaging software (Visitron Systems GmbH). Specimens were illuminated with 561 Cobolt Jive (Cobolt), 405 iBeam Smart, 488 iBeam Smart, and 639 iBeam Smart lasers (Toptica Photonics) and a VS-Homogenizer (Visitron Systems GmbH). All images were acquired with a CFI Plan Apochromat Lambda 100X Oil/1.45 objective (Nikon). This setup results in a pixel size of 130 nm. Unless otherwise indicated, excitation was performed with a 50 ms exposure time in a single plane. The second camera was used to detect the 488 nm channel. To ensure proper camera alignment, TetraSpeck™ Fluorescent Microspheres Size Kit (Thermo Fisher Scientific) was used to image 0.5 μm fluorescent beads after each imaging session. Cells were maintained at 37°C and 5% CO₂ within an incubation box.

2.3 Quantification and Statistical Analysis

Detection of mRNA spots from smFISH and colocalization

Images were registered and maximum intensity projected in all channels. Cells were segmented in two steps. First, a sequential workflow comprising Gaussian filtering steps, Otsu thresholding [23] and distance transform calculations of the DAPI channel (405 nm) were used to segment individual nuclei. Subsequently, the nuclei were used as seeds to perform a watershed segmentation into a previously thresholded cytoplasmic map. Cytoplasmic channels were chosen based on their signal uniformity. SGs were segmented in Fluffy [18]. mRNA spots were detected with a Laplacian of Gaussian. Thresholds for cellular segmentation and spot detection were kept constant throughout a dataset. To colocalize mRNA spots, Euclidean distances were calculated between spots across both channels. To ensure proper channel-to-channel association of mRNA spots, cells with a high spot density were filtered out.

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Cellular outliers with significantly higher or lower nuclear areas, cytoplasmic areas or absolute spot numbers were removed. Two mRNAs were considered as colocalized when their coordinates are less than 2 px (240 nm) apart. In TREAT experiments, all mRNAs in the reference channel are assumed to be intact whereas mRNA counts can vary in the measurement channel. Therefore, cells with low measurement-to-reference pairing were excluded from the analysis.

Processing of live-cell imaging data

Images of the fluorescent beads were used to perform channel alignment. Using the `pystackreg` python package [24] an affine transformation was registered. This model was subsequently re-applied to all images acquired with the secondary camera. Fiji [17] was used to create representative movies and images via cropping, brightness adjustments, channel merging, and scale bar annotation. TrackMate [20] was used to display tracked particles.

Quantification of translational status

Tracking-based quantification of translation was performed using a KNIME [19] workflow described in Mateju et al. [4] in section “Track-based analysis of translational status and colocalization” without including the described SG association component.

Statistics

Results are presented as the mean \pm confidence interval (95%) of independent experiments. Significant differences between variables are based on independent sample t-tests. P-values are indicated using stars in each figure. Each star corresponds to the following p-values:

- ns: $5e^{-2} < p \leq 1$
- *: $e^{-2} < p \leq 5e^{-2}$
- **: $e^{-3} < p \leq e^{-2}$

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- ***: $e^{-4} < p \leq e^{-3}$
- ****: $p \leq e^{-4}$

2.4 Data and Code Availability

The code used to analyze and visualize the data together with plasmid maps is available on GitHub¹. All data supporting the findings of this study are available on reasonable request.

¹<https://github.com/BBQuercus/master-thesis>

3

Results

3.1 The effect of G3BP1 tethering on mRNA dynamics

Proteins of the G3BP RNA-binding family have shown relevance in SGs. In mammals, G3BP1 (and its paralog G3BP2) were reported to be essential in nucleating the formation of SGs [25]. SGs can not only be induced by stress (e.g. sodium arsenite) but also by the overexpression of G3BPs [6]. The exact role of G3BPs in SG assembly as well as in unstressed cells is still largely unknown. As reviewed in Alam and Kenedy [26] various functions have been attributed to this protein family. These range from transcript destabilization and repression to the polar opposite in transcript stabilization. Furthermore, G3BP's also showed effects on transcript localization and sequestration to virus-induced foci. The only consensus is G3BP's involvement in mRNA translational control. The subsequent experiments attempt to clarify this disunity by analyzing G3BP1's effect on mRNA transcripts at a single-molecule level.

This study focussed on one reporter RNA containing a SunTag cassette, the gene encoding for *Renilla* luciferase and MS2 stem-loops (Figure 3.1A). These stem-loops are structural motifs derived from phages and can be specifically recognized by a bacteriophage MS2 coat protein (MCP). G3BP1 was fused to MCP (abbreviated as MCPG3) in an attempt to increase transcript level concentration and improve mRNA recruitment to SGs. The construct was val-

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idated by FISH against the reporter RNA followed by immunofluorescence for the endogenous SG marker TIA-1 [5] (Figure 3.1B). As a control, an identical construct only lacking G3BP1 fusion was used. Both by visual inspection as well as by mRNA quantification (Figure 3.1C), a significant difference in SG association can be seen. MCPG3 shows an around two-fold increase in SG associated mRNAs compared to the control. Taken together, fusing G3BP1 to MCP seems to be a reliable way to recruit functional G3BP1 to the local vicinity of mRNAs.

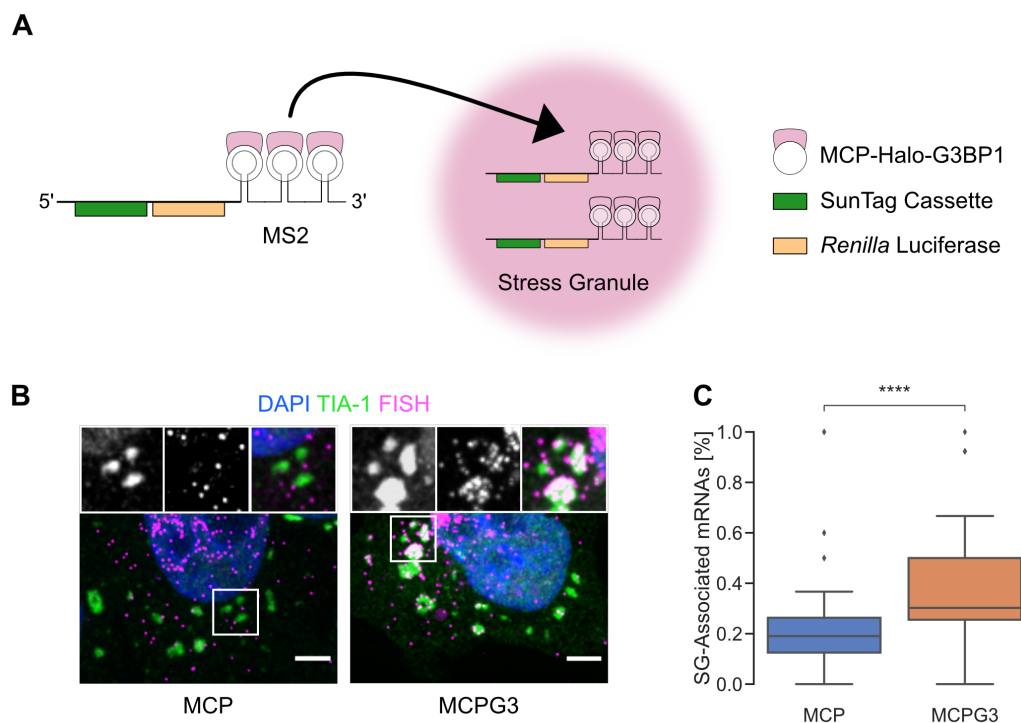


Figure 3.1: Design of MCP-G3BP1. (A) Schematic depiction of MCP-G3BP1 function. (B) Representative fluorescence images of the SG marker TIA-1 and the tethered RNAs. Pannels show TIA-1, FISH, Merge respectively. Scale bars, 10 μ m. (C) Quantification of the mRNA recruitment to SGs. Number of cells quantified (left to right): 27, 21.

3.1.1 Global translational activity

Having proven that G3BP1 can be recruited to mRNAs, I wanted to investigate the effect on translational activity. *Renilla* luciferase assays are used to measure reporter expression on a global level. As G3BP1 was shown to be involved in stress-induced events as well as in unstressed conditions, time points during both induction and stress recovery were measured.

MCPG3 showed a significant increase in luciferase readings in both induction and stress recovery (Figures 3.2A and 3.2B). Interestingly, the largest effects are at later time points and not immediately after stress recovery. This suggests a lesser involvement of G3BP1 on translational activity during stress. SGs have been observed to persist between a few minutes to hours [27]. It is around this time that an increase in luciferase activity can be observed again.

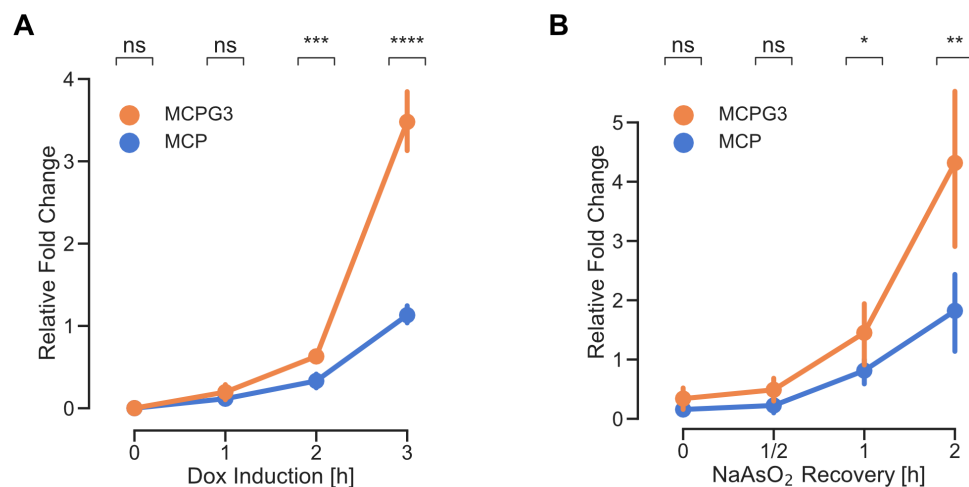


Figure 3.2: G3BP1 tethering increases global translational activity. (A) Luciferase assays at different time points during induction. (B) Sodium arsenite stress recovery time points after induction. (A and B) Data normalized to the 3 hour induction condition of MCP.

3.1.2 Transcript level activity

The observed differences in luciferase activity described in Section 3.1.1 can result from various causes. These translational differences could arrive by an increase in translational activity. The translation site imaging method SunTag (see Section 1.2) can be used to address this option.

In SunTag, the fluorescence intensity is a direct measure of protein production. Therefore, to analyze the translation activity of MCPG3, the track intensity was quantified. Track intensity refers to the average fluorescence intensity over the entire track duration.

During stress conditions, most mRNAs get translationally silenced (reviewed in Holcik and Sonenberg [28]). In order to image translation sites despite this silencing, images were acquired 15 to 30 minutes after removing the stressor.

Analysis of track intensity across entire images shows similar fluorescence intensities (Figure 3.3A). As was to be expected, the translational silencing during stress is not fully recovered leading to lower readings in the recovering cells. Equivalently to the image-level analysis, cellular intensities (Figure 3.3B) do not yield different results. Similarly, the average intensity of all tracks per cell does not appear to be affected by G3BP1 fusion.

Lastly, global translation can also increase by a higher number of translating transcripts per cell. However, as is evident from Figure 3.3C the number of tracks stays consistent in all experiments. This concludes that G3BP1 does not have a profound impact on translation activity.

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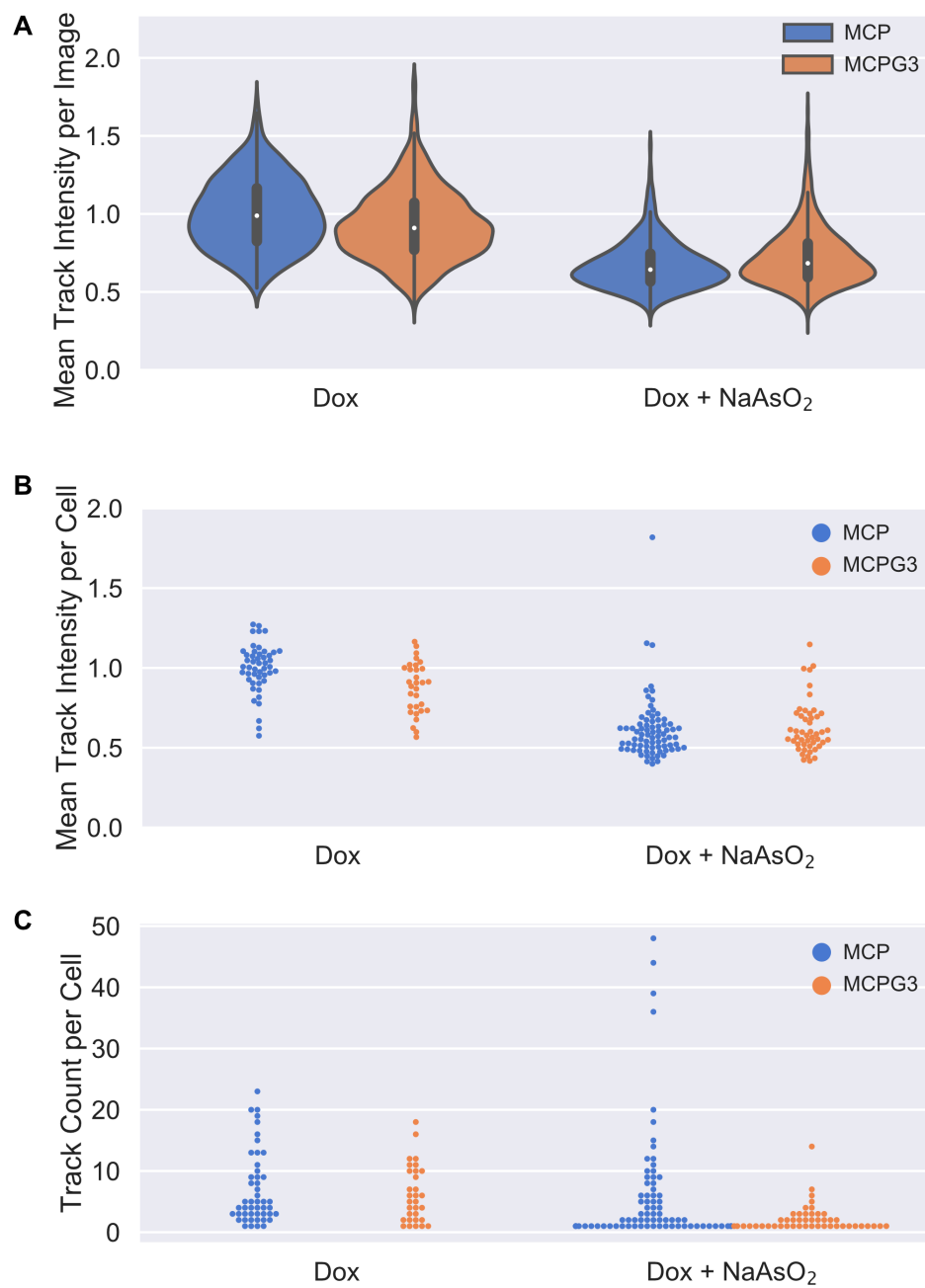


Figure 3.3: (Continued on the following page.)

Figure 3.3: Transcript level imaging of mRNAs. (A) Distribution of the mean fluorescence intensity of tracked mRNAs in the full-sized image (without cellular segmentation). The total number of tracks (left to right): 715, 1064, 997, 3442. (B) Track intensity averaged per cell. (A and B) Data normalized to the Dox induction condition for MCP. (C) The number of tracks registered per cell. (B and C) The total number of cells (left to right): 48, 77, 31, 48.

3.1.3 Transcript level stability

SunTag measurements (see Section 3.1.2) did not suggest any effect of G3BP1 on translational activity. Alternatively, a decrease in mRNA degradation could also explain the original luciferase readings. To ask whether G3BP1 has an mRNA stabilizing effect, I compared the number of intact transcripts between MCPG3 and the control using 3(three)'-RNA end accumulation during turnover (TREAT) [16].

TREAT uses a slightly modified reporter mRNA containing viral RNA pseudo-knot (PK) structures upstream of the MS2 stem-loops. These PKs can block Xrn1 mediated 5'-3' degradation of a transcript by sequestering the 5' phosphate group [29]. Subsequently, by using FISH probes against *Renilla* luciferase upstream of PKs (showing intact mRNAs) and FISH probes against MS2 stem-loops downstream of PKs (showing both intact and degraded mRNAs) one can quantify the number of intact transcripts.

To look at transcript stabilization, five time points were chosen after induction followed by thorough washing to halt any further transcription. As can be seen in Figure 3.4A, transcripts get exported to the cytosol as soon as transcription is stopped.

When comparing the percentage of intact mRNAs, i.e. transcripts still containing FISH spots against both *Renilla* luciferase and MS2, between MCPG3 and the control, a striking difference is evident (Figure 3.4B). The G3BP1 fusion shows a significant increase in intact transcripts compared to the control suggesting an increase in mRNA stability.

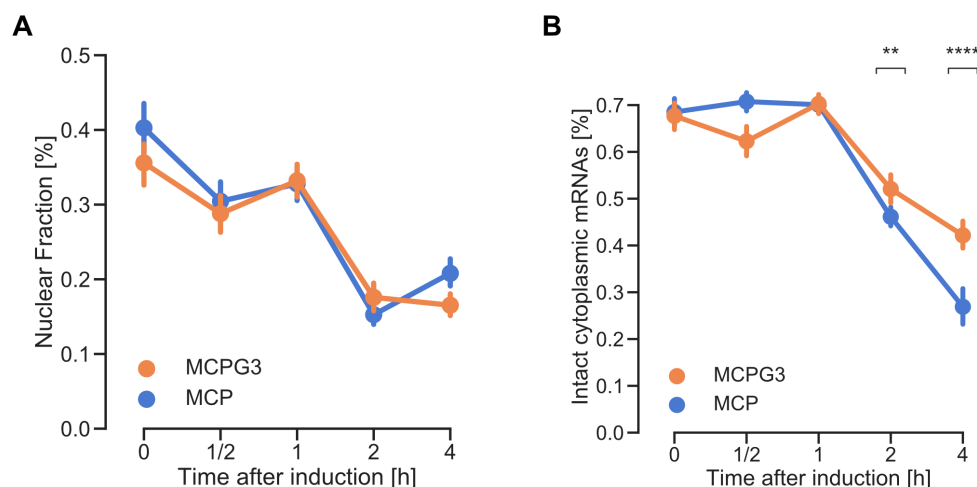


Figure 3.4: TREAT based mRNA stability assay. (A) The fraction of nuclear mRNAs (of total cellular mRNAs) at different time points after induction. (B) Percentage of intact cytoplasmic mRNAs at different time points. (A and B) Number of cells quantified (ascending, MCP then MCPG3): 117, 154, 200, 175, 55, 114, 148, 167, 111, 132.

3.2 Improving the SunTag reporter

The original design of the SunTag cassette consists of 24 GCN4 repeats. These completely disordered repeats are thought to aggregate and could lead to a decrease in translation rates. In an attempt to decrease protein disorder, a spaghetti monster reporter was made. Spaghetti monster green fluorescent proteins (smGFP) are essentially a non-fluorescent green fluorescent protein's (GFP) beta-barrel used to attach small epitope tags. Here, three GCN4 repeats each were placed at the N-terminus, C-terminus, and 10/11-loop of the GFP beta-barrel as shown in Figure 3.5A. Previously, other tags, such as influenza hemagglutinin (HA), were used and visualized with frankenbodies (single-chain antibodies targeting various tags) [30]. To my knowledge, this is the first attempt to combine SunTag imaging with smGFP constructs.

Initially, it was of interest to see whether the smGFP reporter has higher

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translational activity. As is evident from Figure 3.5B, the new reporter shows an approximative 4-fold increase in luciferase activity. A pure *Renilla* reporter was used as control which does not contain any GCN4 repeats. While the smGFP reporter drastically increases activity, it does not yet reach the levels of the control. It must be noted, that the control construct does not contain an FKBP tag which has lowered the activity in previous experiments.

To look at the effect of the new reporter in live cells, stable cell lines were created and observed after 1 hour of induction. From a representative image in Figure 3.5C, one can see that cells typically have more, slightly dimmer translation sites. Quantification of images showed a significant increase in the number of translation sites per cell (Figure 3.5D). The brightness of individual spots decreased around 2.7 fold (Figure 3.5E). This decrease can be explained by a lower number of GCN4 repeats available for the scAB to bind. Whereas the standard SunTag has 24 GCN4 repeats, smGFP only has 9 leading to a binding site difference of $2.6\bar{6}$ to 1.

Taken together, this study proposes a new SunTag reporter which increases translational activity by greatly increasing the number of active transcription sites.

Chapter 3. Results

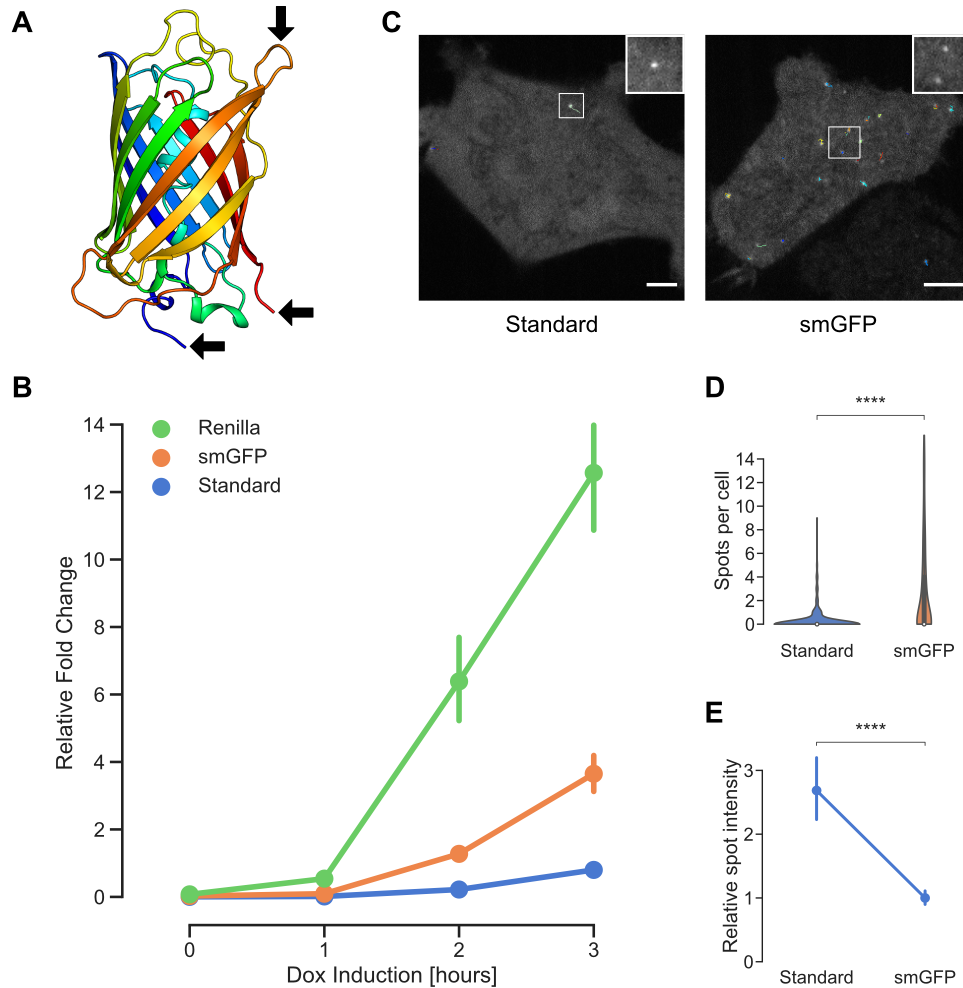


Figure 3.5: Overview of the smGFP SunTag reporter. (A) Structural overview of GCN4 repeat placement on the smGFP beta-barrel (PDB ID: 1GFL [31]). (B) Relative luciferase activity comparing the standard SunTag reporter, the smGFP SunTag reporter, and a Renilla reporter without SunTag cassette. (C) Representative fluorescence images. Each recorded mRNA track is visualized with a unique color. Scale bar, 10 μ m. The inset shows spots from raw images without brightness corrections. (D) Quantification of translation site count per cell. The number of cells quantified (left to right): 286, 233. (E) Average spot intensity normalized to the smGFP reporter. The number of spots quantified (left to right): 36, 39.

4

Discussion

Fusing G3BP1 to MCP has profound effects on their attached mRNAs. First, mRNAs are more likely to get recruited to SGs. The nature of transient transfections used for these sets of experiments likely lowered the number measured if all cells were expressing the construct. Furthermore, due to highly clustered mRNAs in SGs accurate quantification was proven to be rather difficult also decreasing the number. Second, G3BP1 has a significant effect on protein expression irrespective of cellular stress. This interesting finding showed that the role of G3BP1 in SGs might just be secondary while it's more important function occurs in normal cell homeostasis. SunTag imaging experiments were performed to observe differences in translational activity. These, however, were inconclusive and no major differences occurred suggesting that G3BP1 might only have minor roles in translational initiation or elongation. Surprisingly, TREAT assays looking at mRNA stability showed significant differences. This suggests that G3BP1 might actively reduce 5'-3' degradation by sequestering RNase binding sites or inhibiting RNases altogether. Taken together, while some recent publications assume a decay promoting role of G3BP1 [32, 33], others including this study have suggested the opposite [34, 35, 36]. The role of G3BP1 in mRNA metabolism remains debated and needs further clarification on a molecular level.

In an effort to reduce the aggregation potential of the currently used SunTag reporter cassette, a novel cassette relying on the structural support by smGFP was created. The preliminary results shown here suggest that binding affinities are comparable with the previous version while greatly increasing

Chapter 4. Discussion

translational activity. This seems to mainly be achieved through more translating transcripts per cell. The lower signal intensity has shown to make a track based analysis slightly harder. To counteract this, one could, however, use two or more cassettes or add more repeats at other locations within the structural scaffold. Looking forward, it would be interesting to see if mRNA stability is also affected and how the system can be evolved. Recent progress in single-chain antibodies lead the way to a highly interchangeable system with different tags and fluorescent colors.

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Supplementary Methods

NLS-stdMCP-stdHalo-G3BP1 cloning primers:

- gattaggatctatcgattactgccgtggcgcaagccccc
- aagttctgttcagggcccgatggtgatggagaagcctagtcacct
- ctaggcttctccatcaccatcgggccctgaaacagaactt
- gggggcttgcgccacggcagtaaatcgatagatcctaataacctc

smGCN4-Renilla-MS2v5 cloning primers:

- tacacgcggccgcaatcatcggtccgggtggatctggaggtggaggtctggaggagaaga
- cctctttccgagccagttctccctggcccttattccggtttt

Renilla luciferase FISH probes:

- tcgtacaccttgaagccat
- agtgatcatgcgtttgcgtt
- gttcatttgcttcagcgag
- agttgatgaaggagtccagc
- gcgtgcttctcggaatcata
- cagcgttaccatgcagaaaa
- tcgatgtgaggcacgacgtg
- gatgatgcatctagccacgg
- ttaccattccgatcagatc
- gatccaggaggcgatatgag
- caagcggtaggtacttgta
- ttggaagggtcagcagctc

Appendix A. Supplementary Methods

- gtggcccacaaagatgattt
- gagtagtgaaaggccagaca
- ttgatcttgtcttgggtgctc
- cactctcagcatggacgatg
- caggactcgatcacgtccac
- atatcctcctcgatgtcagg
- ctcttcgctcttgatcaggg
- attctcaagcaccattttct
- gcatgggtctcgacgaagaag
- tticcgcgatgatcttgcttg
- cttgaatggctccaggtagg
- gagagggtaggccgtctaac
- ttaacgagagggatctcgcg
- gacaatctggacgacgtcgg
- gaaggtaggcgttgtagttg
- gaacatcttaggcagatcgt
- tggaaaagaaccagggtcg
- cttagctccctcgacaatag
- ttcacgaactcgggtgtagg
- cttacccatttcacatcggag
- gctccacgaagctcttgatg
- tactgctcgttcttcagcac

MS2v4 FISH probes:

- tgccgttttaggtaggatac
- cgcttgaagattggacagtg
- gactgtaatgacagtggagc
- ctgatgctgctggagtttga
- atgtcctgatgtagtcggag
- atcgtcgagcgttgaatgat
- atagtgtctgaggcatgctg
- gtgatcgtgcagccggttga

Appendix A. Supplementary Methods

- cgataacgtagaggcagtag
- ctgatgctcgtcgcagaaga
- atctggtatgtccgatgtg

MS2v5 FISH probes:

- tgattgtgaagtgtcgggtg
- tccacccttgtgtattgtac
- ctgtgtaatgtgtctggagg
- gtgcttctgtttgattgat
- aaatttggtagcagaagccc
- ccttgtgtagttttgag
- cagggaatccctgatggtga
- ccctcgtttgacgtatattg
- gatattcgggaggcgtgatc
- acgcactgaattcgaaagcc
- attcgactctgattggctgc
- ctcttcgcgaaagtcgactt
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- atggagggttgtccagttg
- ttgtctgttggtgagagt
- ctgatgctgcttcgagaaga
- gtatgctcgagtgttcgaa
- gatcgccaccaagaaata
- aattcgtgagagcatgggtg
- tcgtattggacgtggaacga

Appendix A. Supplementary Methods

- tcgtgatcccgaaaggtaag
- agtggaagtgatgtagctgc
- atcgtgcatgcttgaatgtc
- gttgagacttgtggagcatg
- tgaaccatttggtagtttc
- ttgaggtaggagtgggttc
- ttgccagttttgtgggaaga
- ttgggtatgttggaatgggc
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- gtctttcgtatttgtaaacc
- ttgcgctggacgaaagcgtg
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B

Supplementary Figures

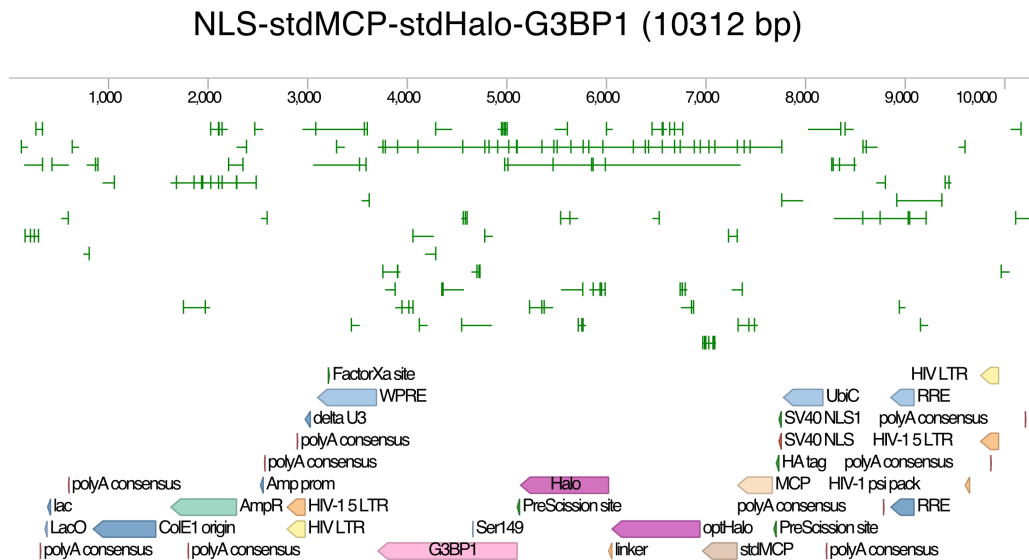


Figure B.1: Design of MCPG3.

Appendix B. Supplementary Figures

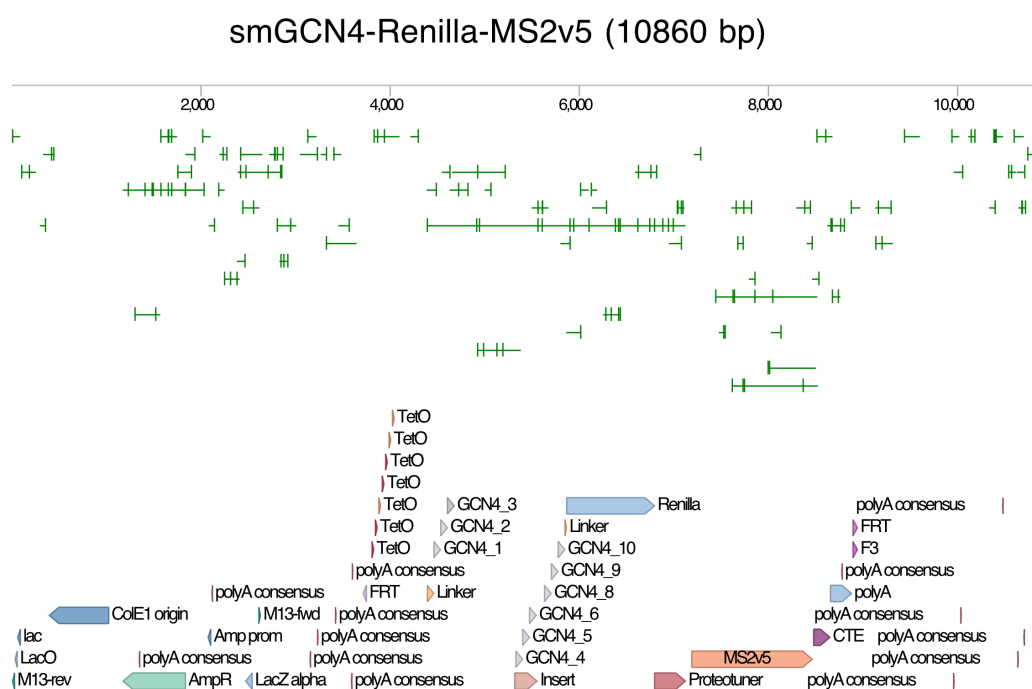


Figure B.2: Design of smGFP SunTag.

Colophon

This document was created using \LaTeX and \BibTeX typesetting originally developed by Leslie Lamport, based on \TeX created by Donald Knuth.

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The text is set in Helvetica.