

1 **Single molecule imaging of the central dogma reveals myosin-2A gene** 2 **expression is regulated by contextual translational buffering**

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11

12 **Abstract**

13 While protein homeostasis is a hallmark of gene regulation, unraveling the hidden
14 regulatory mechanisms that maintain homeostasis is difficult using traditional methods.
15 To confront this problem, we CRISPR engineered a human cell line with multiple tags in
16 the endogenous MYH9 gene, which encodes the essential and ubiquitous myosin-2A
17 cytoskeletal motor. Using these cells, we imaged MYH9 transcription, translation, and
18 mature mRNA and protein in distinct colors, enabling a full dissection of the central
19 dogma. Our data show that MYH9 transcription is upregulated in an SRF-dependent
20 manner in response to cytoskeletal cues and that MYH9 translation can either buffer or
21 match the transcriptional response depending on context. Upon knockdown of actin-
22 depolymerizing proteins like cofilin, translation efficiency drops by a factor of two to buffer
23 strong transcriptional upregulation, likely to help prevent excessive myosin activity. In
24 contrast, following serum stimulation, translation matches the transcriptional response to
25 readily reestablish steady state. Our results identify contextual translational buffering as
26 an important regulatory mechanism driving stable MYH9 expression. They also
27 demonstrate the power and broad applicability of our cell line, which can now be used to
28 accurately quantify central dogma dynamics in response to diverse forms of cellular
29 perturbations.

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33 **Introduction**

34 A grand challenge in cell biology is to image the full central dogma as it plays out in living
35 cells, one molecule at a time. Although tags now exist to separately image individual
36 proteins, mRNA, and sites of transcription and translation ¹⁻⁴, the tags are rarely combined
37 into a single experimental system and even more rarely incorporated into endogenous
38 genes ⁵. This has made it difficult to pinpoint at what level gene regulation occurs ⁶; for
39 example, does a decrease in total protein reflect less translation or more degradation?
40 Alternatively, if levels remain unchanged, does that mean that transcription and
41 translation are also unchanged, or could they instead be changing together, but in
42 opposition?

43 The MYH9 gene exemplifies this challenge. MYH9 encodes non-muscle myosin-2A heavy
44 chain, a motor protein that assembles into bipolar filaments that use ATP to power
45 contraction of the actomyosin cytoskeleton ⁷. This contraction generates intracellular
46 forces that govern cell architecture, migration, adhesion, and division. MYH9 is essential
47 and, like beta-actin, is sometimes categorized as a housekeeping gene, implying stable
48 expression and minimal regulation ^{7, 8}. Consistent with this, myosin activity is thought to
49 be mainly regulated by post-translational phosphorylation, a dynamic and reversible mark
50 that activates myosin motors ⁹. On the other hand, the MYH9 gene has also been
51 categorized as both an oncogene and tumor suppressor ^{10, 11}, and some studies have
52 even shown cancer metastasis relies on MYH9 upregulation ¹². These studies highlight
53 the need to better understand MYH9 regulation and they suggest both too little and too
54 much MYH9 expression is detrimental. This raises an important and generalizable
55 question: do multiple competing regulatory mechanisms strictly enforce the stable
56 expression of genes like MYH9, or does the stability instead arise from a lack of
57 regulation?

58 To directly address this question and better understand how gene expression
59 homeostasis can be established and maintained, we engineered a unique human cell line
60 with multiple tags knocked into the endogenous MYH9 gene. By co-tracking MYH9
61 transcription, translation, and total mRNA at the level of single molecules, we show that
62 translational regulation is a critical aspect of MYH9 expression, one that can be

63 independently regulated from transcription. During cell division or upon the loss of actin
64 depolymerizing proteins, translation buffers transcriptional upregulation, presumably to
65 offset detrimental myosin-2A overexpression. In contrast, upon a release from serum
66 starvation, translation increases in sync with transcription to quickly establish cellular
67 phenotypic changes. Collectively, our results highlight the complexity of the central dogma
68 and provide compelling evidence that even apparently stable genes like MYH9 can hide
69 multitiered and context-dependent gene regulatory mechanisms.

70

71 **Results**

72 ***A system to co-image endogenous transcription and translation with single-*** 73 ***molecule precision***

74 To evaluate gene expression for endogenous MYH9 in human HeLa cells, we performed
75 sequential rounds of CRISPR Cas9 gene editing. First, we incorporated an in-frame C-
76 terminal mClover tag (a GFP variant referred to herein as GFP) at the end of exon 40
77 followed by 24×MS2 stem loops upstream of the native 3' untranslated region. Next, we
78 integrated an N-terminal 6×Flag tagged mCherry after the start codon (**Fig. 1A, S1A**).
79 Following successive rounds of gene editing, we isolated subclones where both MYH9
80 alleles were C-terminally tagged and one was N-terminally tagged (**Fig. 1B**). The tags did
81 not appear to impair MYH9 function because Myo2A-GFP localized to actomyosin
82 structures, including cortical bundles and stress fibers (**Fig. 1C**) and MYH9 mRNA levels
83 were not significantly different than unedited cells (**Fig. S1B**). Using these cells we could
84 visualize mature Myo2A proteins in green (GFP), nascent Myo2A peptide chains at
85 translation sites in red (using Cy3-conjugated anti-Flag antibodies), and Myo2A-encoding
86 mRNA in far-red (using Halo-tagged MS2 coat protein with JF646 ligand), enabling a full
87 dissection of the central dogma in separate colors. Hereafter we refer to this cell line as
88 eMyo2AGFP.

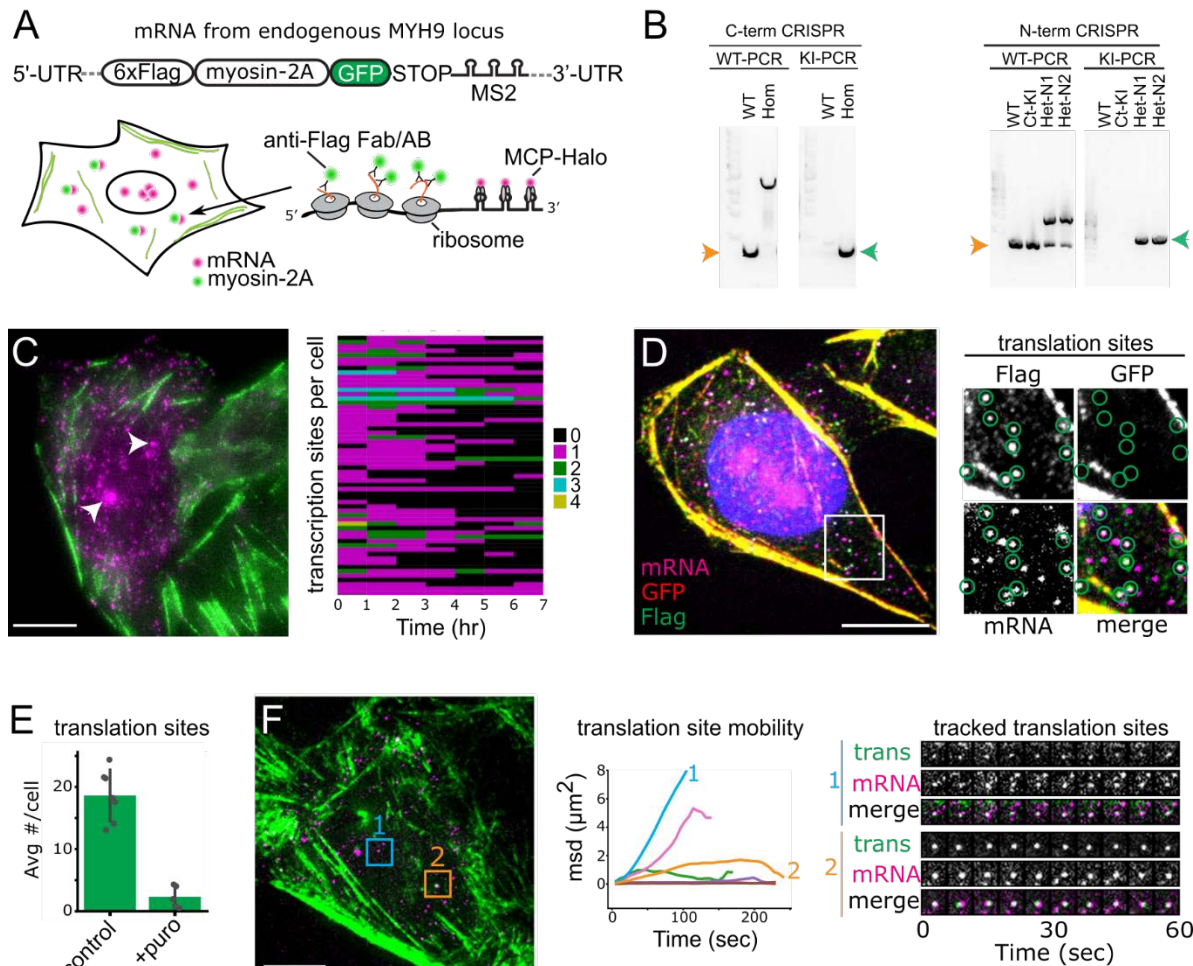


Fig. 1 A cell line for dissecting the central dogma with single-molecule precision. **A.** Top, schematic of a tagged MYH9 transcript in CRISPR modified eMyo2AGFP cells. Below, cartoon of an eMyo2AGFP cell expressing myosin2A-encoding mRNA (magenta), myosin2A protein (green) and translation sites (colocalized foci of mRNA and protein). **B.** Genomic PCR for detection of wild type (orange arrow) and knock-in (green arrow, homo or heterozygous) alleles, following successive rounds of CRISPR editing. **C.** Live cell image of eMyo2AGFP cell with myo2A-GFP protein (green) and mRNA (magenta), arrowheads show mRNA transcription foci. Heatmap of numbers of transcription sites over 1hr intervals, each row corresponds to one cell (right). **D.** Representative confocal fixed cell image showing myo2A mRNAs without translation signals (magenta), and translating mRNAs marked by colocalized mRNA and Flag-myosin-2A (green) but absence of mature myo2A-GFP (red). Boxed region is enlarged (right) with translation sites encircled. **E.** Quantification of loss of translation sites following puromycin treatment. Mean \pm SD, 580 cells control and 285 cells puro. **F.** Live cell confocal image of anti-FLAG Fab labeled Flag-myosin-2A (green) and mRNA (magenta). Mean squared displacement (msd) plots for 7 mRNA tracks (center), including translation site mobility of a single mRNA (1) or an mRNA cluster (2) for boxed regions also shown in time series images, right. Scale bars 10 μ m.

89 To demonstrate our ability to detect both transcription and translation with single molecule
 90 sensitivity in eMyo2AGFP cells, we first focused on steady-state MYH9 expression. Live
 91 imaging revealed bursts of transcription at between one and four sites per cell (**Fig. 1C**
 92 **and Movie S1**), as would be expected for a diploid locus. Cells had tens to hundreds of
 93 MYH9 transcripts in the nucleus and cytoplasm (**Fig. 1C**). To identify which were
 94 translated, we immunostained fixed cells and looked for bright FLAG signals that

95 overlapped with mRNA yet lacked GFP (since GFP takes time to mature after translation).
96 According to this metric, many cytoplasmic mRNA were translation sites (**Fig. 1D**) and
97 these reassuringly disappeared upon treatment with the translational inhibitor puromycin
98 (**Fig. 1E**).

99 To further characterize individual Myo2A translation sites, we performed live imaging with
100 anti-FLAG intrabodies ⁴. This revealed diverse behaviors, from fast unidirectional
101 movement to slow, nearly immobile diffusion (**Fig. 1F and Movie S2**). We usually
102 detected translation from single mRNAs, although a small fraction (<1%) were in bright
103 clusters, reminiscent of translation factories (**Fig. 1F, spot 2**) (ref 13). Altogether, our data
104 demonstrate eMyo2AGFP cells are versatile tools to comprehensively examine broad
105 aspects of MYH9 gene expression with single molecule precision.

106

107 ***MYH9 translation efficiency is high and undergoes regulation during cell division***

108 Confident in our ability to detect individual mRNA and translation sites, we next set out to
109 quantify their average numbers in cells. We used fixed cells for this analysis because we
110 obtained better signal to noise and tag saturation than is possible in live cells. On average,
111 eMyo2AGFP cells contained 90 ± 47 total mRNA per cell (**Fig. 2A**). Of these, 53 ± 29
112 were in the cytoplasm, 20 ± 10 of which were being translated (we did not detect nuclear
113 translation). Separately, the fraction of cytoplasmic mRNAs translating the FLAG tag was
114 measured at $46 \pm 6\%$ (**Fig. 2B**). Since we only expect half of the cytoplasmic transcripts
115 to encode the FLAG tag in the first place (due to its heterozygous insertion), we estimate
116 the translation efficiency to be double our measured cytoplasmic fraction $\sim 93\%$ (**Fig. 2B,**
117 **projected**). These data therefore suggest that the majority of cytoplasmic MYH9
118 transcripts were being translated.

119 The high efficiency of MYH9 translation is perhaps not surprising given the abundance of
120 myosin-2A required to form the actomyosin cytoskeleton. However, this left us wondering

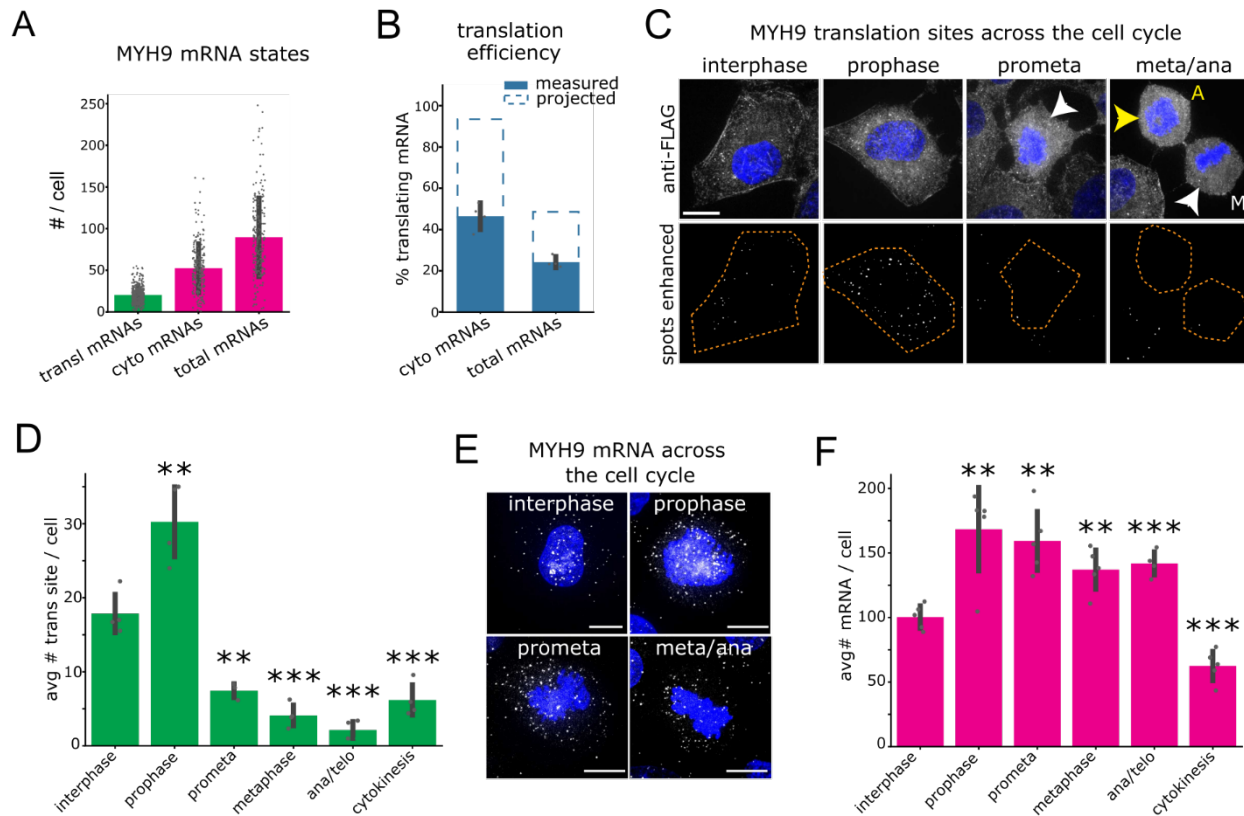


Fig. 2 MYH9 translation is downregulated during cell division. **A.** Quantification of mRNAs and translation. Mean \pm SD. **B.** Quantification of translating mRNA percentage. Note, in eMyo2AGFP cells there are two labeled MYH9 mRNA alleles, of which only one detects translation (measured). Mean \pm SD, $n = 361$ cells. Dashed line (projected) shows projected % translation accounting for two alleles. **C.** Representative confocal immunofluorescence images of MYH9 translation sites labeled by anti-Flag and for DNA (blue). A-anaphase cell, M-metaphase cell. Lower panels show spot fluorescence enhanced by a LOG filter for outlined cells. **D.** Quantification of translation sites across the cell cycle. Mean \pm SD, interphase $n = 312$ cells, mitotic $n \geq 20$ cells/phase. **E.** Fluorescence images of MYH9 mRNAs labeled by Halo-MCP were imaged in fixed cells at different stages of the cell cycle (DNA in blue). **F.** Quantification of the average number of mRNA per cell. Mean \pm SD, interphase $n = 122$ cells, mitotic $n \geq 18$ cells/phase. Scale bars 10 μm . ** $p \leq 0.01$, *** $p \leq 0.001$, Welch's t-test.

121 if MYH9 translation is subject to regulation. To test this, we imaged eMyo2AGFP cells
 122 across the cell cycle (**Fig. 2C**), avoiding the use of drugs commonly used to synchronize
 123 cells (which can suffer from artifacts¹⁴). We hypothesized translation might be modulated
 124 during cell division since myosin-2 motors are known to play an important and highly
 125 conserved role in contractile ring assembly. Our analysis confirmed this: we observed a
 126 sharp increase in the number of translation sites at prophase relative to interphase,
 127 followed by a dramatic drop from prometaphase through cytokinesis (**Fig. 2D**).
 128 Interestingly, while the initial increase could be explained by a commensurate increase in
 129 MYH9 mRNA levels, the later drop did not follow the mRNA pattern (**Fig. 2E, F**). Thus, it

130 appears that MYH9 translation is downregulated independently from transcription just
131 after prophase.

132 Rounded mitotic cells generally have reduced cell surface area relative to those of
133 interphase (**Fig. S2A**) and there was a strong positive correlation between cell size and
134 MYH9 translation (**Fig. S2B**), so we explored whether cell size alterations could account
135 for the reduced translation. However, normalization of MYH9 translation sites to cell area
136 still showed reduced MYH9 translation for cells in prometaphase onwards (**Fig. S2C**). We
137 conclude that reduced mitotic MYH9 translation is not a function of decreased cell areas.
138 In summary, our results identify existence of a post-transcriptional mechanism that
139 modulates the overall high efficiency of MYH9 mRNA translation in a cell-cycle dependent
140 manner.

141 ***Silencing of actin depolymerizing proteins stimulates SRF-dependent MYH9*** 142 ***transcriptional bursts***

143 Having characterized MYH9 expression in steady state, we now turned our attention to
144 MYH9 stimulation. In an earlier study we showed that knockdown of two closely related

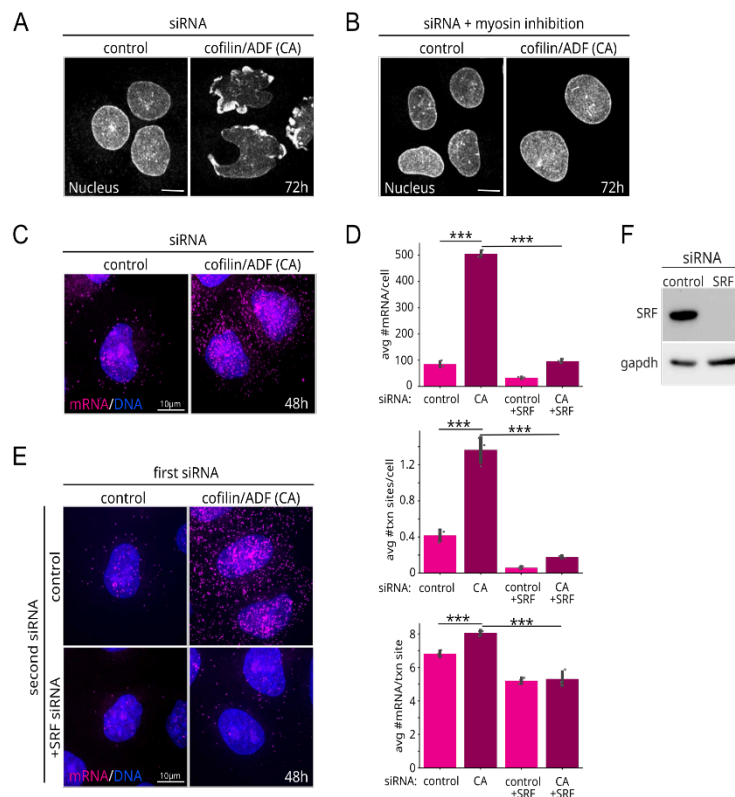


Fig. 3 SRF-dependent MYH9 transcriptional bursts in response to knock-down of actin polymerizing proteins. **A, B.** Representative immunofluorescence images of nuclear envelope labeled cells depicting nuclear dysmorphology induced by cofilin/ADF silencing (A) and rescue by myosin inhibition via Y27632 treatment (B). **C-E.** MCP-Halo fluorescence images of MYH9 mRNA (magenta, C and E) and quantification of MYH9 mRNAs (D, top), transcription sites (D, middle) and burst amplitude (D, bottom) following cofilin/ADF silencing (C) and co-silencing of SRF (E). Scale bars 10 μ m. Values are mean \pm SD, $n \geq 186$ cells/treatment. *** $p \leq 0.001$, Welch's t-test. **F.** Immunoblot of SRF siRNA silencing.

145 actin depolymerizing proteins, cofilin and ADF, results in detrimental myosin contractile
146 activity¹⁵. Over a period of 72 hours, this leads to excessive forces in cells that contort
147 nuclei into tortured shapes that are reminiscent of those founds in tumors (**Fig. 3A**). This
148 detrimental phenotype can be rescued by co-inhibiting myosin during cofilin/ADF
149 silencing, demonstrating the phenotype is due to excessive myosin-based forces (**Fig.**
150 **3B, and ref 16** for quantification). We previously showed this is in part due to enhanced
151 association of myosin with F-actin (since cofilin competes with myosin for F-actin).
152 However, *in vivo* studies of ADF null mice suggested a link between defective actin
153 dynamics and actomyosin gene expression¹⁷. This led us to ask if deregulated MYH9
154 expression was also partly responsible.

155 To test this, we silenced cofilin/ADF expression using siRNA in eMyo2AGFP cells (**Fig.**
156 **3C**). Consistent with MYH9 deregulation, the number of MYH9 mRNA per cell
157 dramatically increased from ~100 to ~500 after 48 hours (**Fig. 3D, top, Control & CA**;
158 note at 72 h, when nuclei are contorted, there were too many mRNA to count). The mRNA
159 increase was caused by a roughly 3-fold increase in the number of transcription sites per
160 cell (burst frequency; **Fig. 3D, middle, Control & CA**) in combination with a modest
161 increase of ~1.3 mRNA per transcription site (burst amplitude; **Fig. 3D, bottom, Control**
162 **vs CA**). Live-cell analysis confirmed increased temporal MYH9 burst activity for
163 cofilin/ADF silenced cells (**Fig. S3C**). To see if there were additional changes to
164 transcription kinetics after an allele is activated, we tracked active sites over a period of
165 2-5 hours (**Fig. S3A and Movie S3**). However, classification of their fluctuations using a
166 three-state Hidden Markov Model^{18, 19} revealed no significant changes (**Fig. S3B**). It
167 remains possible that measurements over periods longer than made for our study, would
168 reveal changes to heterogenous long refractory transcriptional OFF times, as suggested
169 by a recent study²⁰. A likelihood, given we detected some cells with extended OFF
170 intervals (**Fig. S3C**). Together, these data suggest the major impact of cofilin/ADF
171 knockdown on MYH9 transcription is enhanced activation.

172 Stimulation of transcription via simultaneous frequency and amplitude modulation is not
173 universal²¹, but has been observed for genes such as beta-actin²², whose transcription
174 is linked to a master cytoskeleton transcription factor, Serum Response Factor (SRF)²³.

175 As MYH9 is also a putative target of SRF, we wondered if SRF is responsible for the
 176 enhanced transcription. To see if this was the case, we repeated our cofilin/ADF
 177 knockdown experiments, but in addition we co-silenced SRF (**Fig. 3E, F**). Remarkably,
 178 this almost completely blocked the increase in MYH9 mRNA levels, attenuating both burst
 179 frequency and amplitude (**Fig. 3D, Control+SRF & CA+SRF**). These results identify SRF
 180 as an essential mediator of transcription burst dynamics for the MYH9 gene in both basal
 181 and stimulated conditions. The data also argue that elevated MYH9 mRNA levels
 182 following cofilin/ADF loss results from increased transcription and not to other

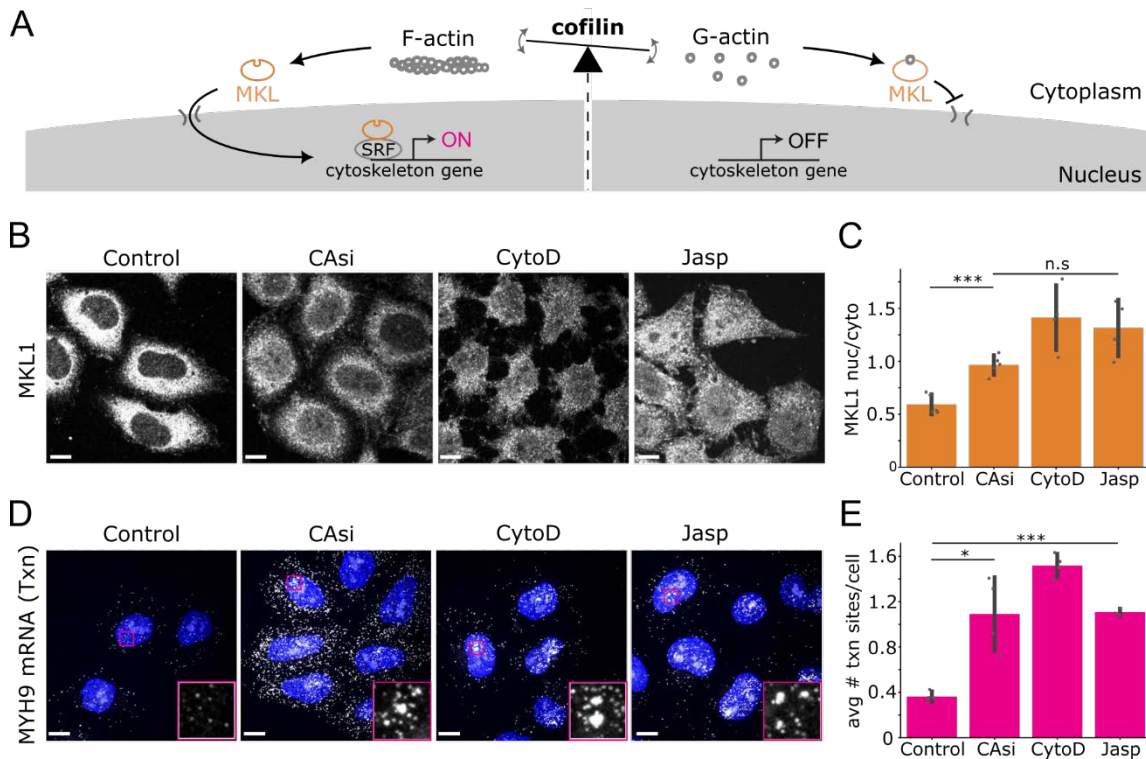


Fig. 4 Altered cytoskeletal dynamics promote MKL1 nuclear localization and MYH9 transcriptional bursts. A. Illustration of model by which cofilin activity and actin dynamics may regulate MKL1 localization and SRF-dependent cytoskeletal gene expression. **B and C.** Nuclear level confocal immunofluorescence images (B) and quantification (C) of MKL1 nuclear/cytoplasmic distribution for cells treated with cofilin/ADF siRNAs (CAsi), cytochalasin D (cytoD) and Jasplakinolide (Jasp). **D and E.** Confocal fluorescence images (D) and quantification (E) of MCP-Halo labeled MYH9 mRNAs. DNA in blue. Insets (D) show enlargement of boxed regions depicting transcription sites. Note, treatment periods are 48h for siRNAs and 2h for drugs. Scale bars 10 μ m. Values are mean \pm SD, $n \geq 350$ cells/treatment (C), $n \geq 330$ cells/treatment (E). * $p < 0.05$, *** $p \leq 0.001$, n.s not significant by Welch's t-test.

183 possibilities, such as mRNA stabilization.

184

185 ***MYH9 transcription is linked to cytoskeletal signaling that promote MKL1 nuclear***
 186 ***localization***

187 By what mechanism does loss of cofilin/ADF lead to SRF-dependent MYH9 transcription?
188 This is an important question because cofilin inactivation, just like myosin-2A
189 overexpression, has been linked to cancer ²⁴. Transcription of cytoskeletal genes by SRF
190 relies on MKL-family cofactors (a.k.a. MAL, MRTF, ref 23). According to a model (**Fig.**
191 **4A**), when G-actin subunits assemble into actin filaments (F-actin), MKL can enter the
192 nucleus with SRF and activate cytoskeletal genes. However, if G-actin levels become
193 sufficiently high, they bind MKL and prevent its translocation into the nucleus, causing
194 attenuation of cytoskeletal gene expression. This cascade ostensibly creates a self-
195 regulatory feedback loop, dictated by the balance of G- to F-actin.

196 With this model in mind, since cofilin and ADF both disassemble actin filaments into G-
197 actin, their loss should lower G-actin levels, causing MKL to translocate into the nucleus
198 and activate cytoskeletal genes like MYH9. To test this model, we measured MKL1 levels
199 in eMyo2AGFP cells at 48 hours post cofilin/ADF silencing and indeed found a significant
200 increase in the nuclear fraction of MKL1 (**Fig. 4B, C**). However, the increase was not
201 particularly striking, as the majority of MKL1 remained in the cytoplasm. We saw a similar
202 result in a set of related experiments in which we promoted levels of MKL unbound by G-
203 actin using drugs (cytochalasin D displaces G-actin from MKL and jasplakinolide
204 stabilizes F-actin ²⁵, reducing G-actin) (**Fig. 4B, C**). In all cases, MKL1 nuclear localization
205 was only modest despite a robust SRF-dependent MYH9 transcriptional response (**Fig.**
206 **4D, E**). Our data therefore point to a feedback mechanism between actin cytoskeletal
207 organization and MYH9 transcription and suggest that the threshold of nuclear MKL1
208 levels necessary for activation of endogenous SRF/MKL targets may be lower than
209 commonly expected.

210

211 ***MYH9 expression is stabilized by a translational buffering system***

212 Given the fact that persistent MYH9 upregulation leads to excessive intracellular forces
213 that contort cell nuclei and possibly contributes to cancer, we wondered if there were any
214 post-transcriptional expression mechanisms that might counter the strong transcriptional
215 induction seen after cofilin/ADF silencing. As a first check, we plotted total myosin-2A
216 protein levels versus the number of cytoplasmic mRNA in eMyo2AGFP cells. This

217 revealed a positive correlation in control conditions, but only a weak correlation after
 218 cofilin/ADF silencing (Pearson $r = 0.61$ vs 0.11 , **Fig. 5A**). Regression slopes indicated a
 219 reduced rate of change of MYH9 protein to mRNA (slope = 2.3×10^7 vs 2.9×10^6),
 220 suggesting some form of protein buffering, either at the level of mRNA translation or
 221 protein decay.

222 To see which of these was at play, we quantified the number and intensity of translation
 223 sites in control and cofilin/ADF silenced cells. Knockdown of cofilin/ADF resulted in a 3.5-
 224 fold increase in MYH9 translation sites (**Fig. 5B, C**). Consistent with translational
 225 buffering, the fraction of cytoplasmic mRNA that were being translated was reduced by a
 226 factor of more than two (**Fig. 5D**). We did not detect a similar decrease in translation
 227 efficiency for a control translation reporter, HA-KDM5B-MS2 (**Fig. 5D**), suggesting the
 228 reduction in translation efficiency is not global. Interestingly, although we expected the

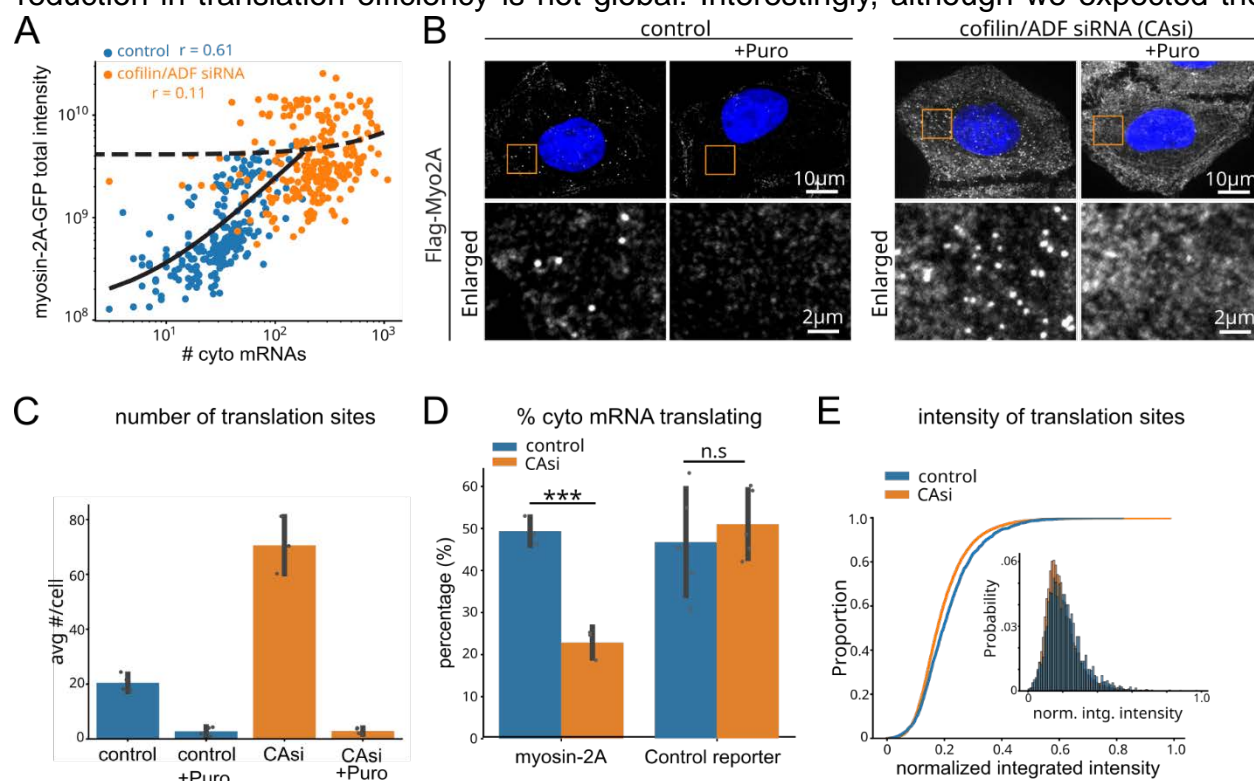


Fig. 5 Depletion of actin depolymerizing proteins results in MYH9 translational buffering. **A.** Log scale correlation plot of per cell cytoplasmic MYH9 mRNAs vs total myosin-2A-GFP intensity with control or cofilin/ADF siRNA (CA) treatments. **B and C.** Confocal immunofluorescence images (B) and quantification (C) of anti-Flag labeled MYH9 translation sites. Boxed regions are shown in enlargements. Values are mean \pm SD, $n \geq 201$ cells/treatment. **D.** Proportion of translating myosin-2A or KDM5B control mRNAs. Mean \pm SD, $n \geq 209$ cells/treatment. **E.** Cumulative distribution functions and histogram (inset) of normalized translation site intensity, control $n=165$ cells and 2098 translation sites, cofilin/ADF siRNA (CA) $n = 174$ cells and 7272 translation sites. *** $p \leq 0.001$, n.s., not significant, Welch's t-test.

229 intensity of individual MYH9 translation sites to also be reduced, this was not the case
 230 (**Fig. 5E**). Instead, their intensities remained similar, suggesting whatever form MYH9
 231 translational buffering takes, it operates more like an on/off switch than a dimmer. In
 232 conclusion, our data demonstrate MYH9 is regulated by a translational buffering system
 233 that stabilizes MYH9 expression under conditions of persistent transcriptional
 234 upregulation, presumably to delay the detrimental effects of myosin-2A overexpression.

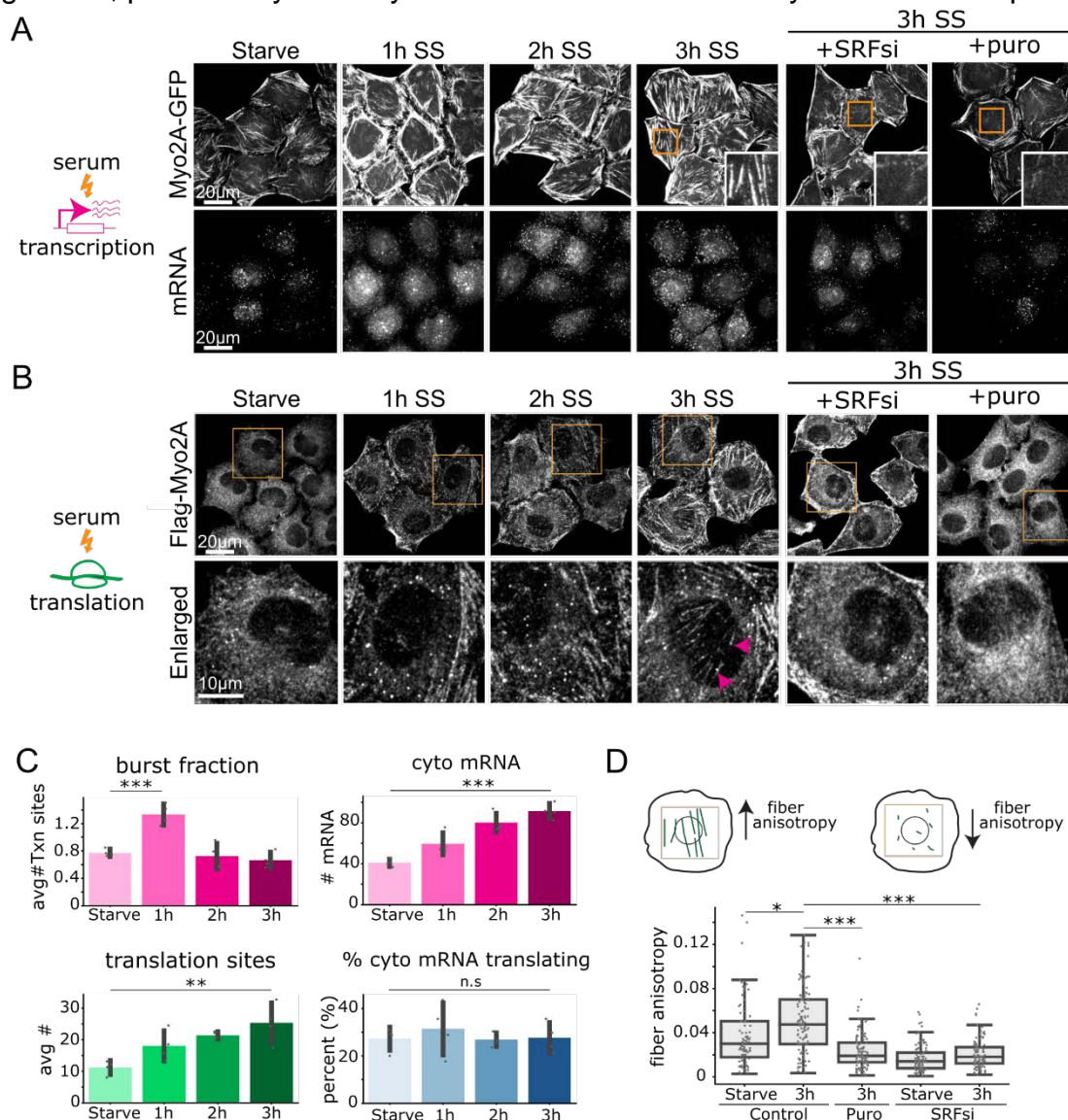


Fig. 6 Transcription and translation work together to establish cellular phenotype upon serum stimulation. A. Confocal fluorescence images depicting actomyosin cytoskeletal rearrangements and MYH9 mRNAs following serum stimulation (SS). Boxed regions, shown enlarged in insets, exemplify loss of central stress fibers at 3h SS following SRF siRNA or puromycin treatments. **B.** Immunofluorescence images of anti-Flag labeled myosin-2A translation. Boxed regions are shown enlarged in lower panels. Note presence of translation spots associated with a central stress fiber (3h SS, magenta arrowheads). **C.** Quantification of transcription and translation features, values are mean \pm SD, $n \geq 156$ cells/treatment. **D.** Illustration and measurements of central stress fiber ordering. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s., not significant, Welch's t-test. See also Fig. S4.

235 ***MYH9 transcription and translation cooperate upon serum stimulus and are both***
236 ***required for actomyosin remodeling***

237 Given our previous results we wondered if translational buffering is a universal feature of
238 MYH9 expression. Growth factor stimulation produces rapid (< 30 min) actomyosin
239 cytoskeletal reorganization concurrent with SRF transcriptional activation²⁶⁻²⁸. How
240 transcription is coupled with translation to this physiologic cue, and whether new protein
241 synthesis may contribute to the near-term cytoskeletal response remain unknown.

242 To address these questions, we serum starved eMyo2AGFP cells and then re-exposed
243 them to serum, measuring total MYH9 mRNA and protein production (**Fig. 6A, B**). This
244 led to rapid actomyosin rearrangements, including the formation of circumferential
245 actomyosin bundles after one hour and an increase in the numbers of ventral stress fibers
246 over the central portion of cells over a period of three hours (**Fig. 6A**). Serum stimulation
247 produced a transient elevation of MYH9 transcription that peaked at 1h post stimulation,
248 as evidenced by an increase in the burst fraction (**Fig. 6C, S4D**). Unlike the translational
249 buffering we observed upon cofilin/ADF silencing, serum stimulation did not significantly
250 alter the fraction of translating cytoplasmic mRNAs (**Fig. 6C**). Instead, the numbers of
251 MYH9 translation sites continued to increase in tandem with the number of cytoplasmic
252 mRNA (**Fig. 6C, S4G**). Furthermore, a modest positive linear correlation between MYH9
253 protein and mRNA levels was maintained at this post-stimulus timepoint (**Fig. S4A**,
254 Pearson $r = 0.37$ vs 0.43). Altogether, these data suggest that MYH9 translation does not
255 always counter the transcriptional response, implying translational buffering is context-
256 dependent.

257 Since serum stimulation activates MYH9 transcription, we evaluated whether SRF-
258 dependent transcription and new protein synthesis contributed to actomyosin cytoskeletal
259 reorganization following this stimulation. Transient MYH9 transcriptional activation, and
260 increased levels of its mRNAs and translation sites were all abrogated by silencing of
261 SRF (**Fig. S4C-F**). Notably, while SRF silencing or inhibition of translation with puromycin
262 did not prevent formation of peripheral actomyosin bundles at 1h post serum stimulation
263 (**Fig. S4C**), both treatments disrupted formation of stress fibers over the central portion
264 of cells by 3h post serum stimulation (**Fig. 6A, B**), resulting in reduced linear fiber ordering

265 **(Fig. 6D)**. These results suggest that spatial actomyosin stress fiber assembly, within a
266 window of 3h following growth factor stimulus, requires both SRF-dependent transcription
267 and new protein synthesis. Furthermore, the presence of MYH9 translation sites along
268 central stress fibers at 3h post stimulation (**Fig. 6B**, magenta arrowheads) is consistent
269 with a model of localized MYH9 protein synthesis contributing to assembly of these
270 structures. Interestingly, while total levels of MYH9 protein increased marginally following
271 serum stimulation, this remained below statistical significance at the 3h timepoint (**Fig.**
272 **S4B**). This result is also in accord with a conclusion that new MYH9 protein synthesis
273 may not be required to support global actomyosin activity but rather to localized functions.
274 Changes to myosin-2A protein turnover during this period could contribute to such an
275 outcome.

276

277 **Discussion**

278 Homeostatic maintenance of protein levels is an overarching feature of gene expression,
279 but the hidden regulatory mechanisms that maintain homeostasis have been difficult to
280 study and verify. By engineering a novel cell line in which all aspects of MYH9 activity can
281 be visualized in separate colors and with single-molecule precision, we discovered a
282 highly dynamic and multilayered regulatory network lying beneath the apparent stability
283 of myosin-2A expression.

284 Much like a thermostat maintains temperature, our study provides evidence that MYH9
285 translation is a key regulatory checkpoint that ensures myosin-2A levels are acceptable.
286 In steady state, nearly every cytoplasmic MYH9 mRNA is translated to meet the demands
287 of the actomyosin cytoskeleton. However, in scenarios when myosin-2A levels become
288 too high, such as when transcription is persistently elevated upon loss of actin
289 depolymerizing proteins like cofilin, translation efficiency is down regulated. Such
290 translational buffering stabilizes protein production to minimize the damaging effects of
291 excessive myosin. Similarly, during cell division, MYH9 translational buffering counters a
292 sharp transcriptional burst. Specific lowering of MYH9 translation during mitosis may
293 safeguard against defects such as impaired karyokinesis, as observed in cofilin-depleted
294 cells with excessive myosin activation ¹⁵. Conversely, in scenarios that elevate

295 actomyosin assembly, such as the recovery from serum starvation, MYH9 translation
296 aligns with transcription. This is necessary to rapidly remodel the actomyosin cytoskeleton
297 so cellular steady state can be quickly reestablished. Thus, depending on the precise
298 cellular context, our data argue MYH9 translational regulation can either facilitate or buffer
299 the transcriptional response to help ensure myosin-2A levels stay within an acceptable
300 range.

301 While we were unable to pinpoint the precise molecular mechanism behind MYH9
302 translational buffering, our study offers some clues. First, the default mode of MYH9
303 translation seems to be the ON state, since the vast majority of transcripts appear to be
304 translated throughout interphase. Second, the observation that buffering can more than
305 halve the fraction of translating cytoplasmic MYH9 mRNA while leaving their intensities
306 relatively unchanged suggests buffering acts more like a binary on/off switch than a
307 gradual fader. These observation hint at the possible involvement of specific RNA-binding
308 proteins or post-transcriptional modifications that selectively silence MYH9 mRNA.
309 Identifying these specific factors will be an interesting avenue of future research, although
310 a major challenge will be sorting myosin-specific regulators from other targets of the broad
311 SRF/MKL nexus ²⁹.

312 A striking finding of our study is that a just-in-time mode of local protein synthesis appears
313 necessary for assembly of certain actomyosin stress fibers within a 3h period following
314 growth factor stimulation. This result was surprising for at least two reasons. First, growth
315 factor signaling produces rapid stress fiber assembly within 20 minutes of stimulus,
316 suggesting transcription and global new protein synthesis are not required for immediate
317 actomyosin remodeling^{26, 27}. Consistent with this view, peripheral actomyosin bundling
318 within 1h of serum stimulus was not lost by perturbations to MYH9 transcription or through
319 protein synthesis inhibition. Secondly, a requirement for new protein synthesis occurred
320 during a period where total MYH9 protein levels did not change significantly. As such, bulk
321 level assays of protein amounts would not have foreshadowed a need for new MYH9
322 protein synthesis. Nevertheless, loss of central stress fibers at 3h post stimulus, through
323 either inhibition of SRF transcription or protein synthesis, suggested a requirement for
324 both new mRNA transcripts and local translation, similar to what has been observed for

325 actin³⁰. De novo mRNA and protein synthesis of MYH9 and other SRF targets may
326 therefore be a means to specify a distinct population of molecules, without previous
327 modifications, for current localized use.

328 At the level of transcription, our study sheds light on the elusive molecular pathways that
329 link cofilin inactivation and myosin overactivity. Central to this pathway is MKL, a
330 transcription factor that activates MYH9 via SRF but whose translocation into the nucleus
331 is inhibited by G-actin. According to our data, depletion of the actin depolymerizing
332 proteins cofilin and ADF lowers G-actin levels, leading to MKL nuclear translocation and
333 enhanced transcriptional activation of MYH9. Left unchecked, this leads to excessive
334 myosin contractile forces that, despite the presence of translational buffering, eventually
335 lead to severe morphological aberrations in cells and potentially oncogenic
336 transformations³¹. Our combined data therefore provide a compelling molecular pathway
337 that would link cofilin-inactivation, higher myosin expression and activity, translational
338 buffering, and disease states.

339 Having demonstrated the potential of eMyo2AGFP cells, we envision many possible
340 applications. They hold especially great promise for screening purposes, where they can
341 help uncover hidden gene regulatory dynamics. While powerful, there are several caveats
342 to their use worth highlighting. First, it can be technically challenging to detect dimmer
343 myosin-2A translation sites due to the abundance of background signal from mature
344 myosin-2A. While we used the lack of a GFP signal to distinguish translation sites from
345 mature structures, the high background could still be problematic. We therefore
346 recommend measurements of translation efficiency be made in fixed rather than live cells,
347 where FLAG binding sites can be fully saturated and background signal minimized.
348 Second, although we were able to insert mRNA and GFP tags into both MYH9 alleles, we
349 only succeeded in inserting FLAG tags into one allele. This leaves a hidden translation
350 fraction in eMyo2AGFP cells that can only be indirectly assessed. While we believe the
351 hidden fraction behaves similarly to the tagged fraction since we detected translation from
352 nearly half the mRNA, we cannot rule out the possibility that the hidden fraction behaves
353 differently. Third, it remains unclear to what extent our approach can be generalized to
354 other cell lines or other genes. Some cells may be difficult to CRISPR edit and some

355 genes may not tolerate tagging. Keeping these caveats in mind, we believe eMyo2AGFP
356 cells represent a significant advance and should be of broad interest to those wishing to
357 explore the intricacies of gene regulation in the context of cellular perturbations of any
358 kind.

359

360 **Methods**

361 *Cell Culture and Drug Treatments*

362 Human HeLa-kyoto cells were obtained from ATCC and maintained in high glucose
363 DMEM supplemented with 10% fetal bovine serum (Atlas). For translation inhibition, cells
364 were treated at 50 μ g/ml with puromycin (Sigma) for 20 min or as otherwise indicated.
365 Cells for MKL nuclear-cytoplasmic assessment were treated with 2 μ M cytochalasin D
366 (Cayman Chemical), 1 μ M Jasplakinolide (Cayman Chemical) or dimethyl sulfoxide
367 (Sigma) for controls, for 2h prior to fixation for analyses. For serum stimulation
368 experiments cells were starved for 18-24 h in medium containing 0.3% serum and
369 stimulated by replacing with medium containing 15% serum.

370 *Gene Editing*

371 Sequential rounds of CRISPR/Cas9 gene editing of the endogenous MYH9 gene locus
372 located on chromosome 22 was performed as previously detailed ³². Briefly, guide RNAs
373 targeting the start ATG or stop codons of coding exons 1 and 40, respectively, were cloned
374 into plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9. For C-terminal tagging a homology
375 directed repair donor plasmid containing mClover3 (derived from Addgene plasmid
376 72829), followed by 24xMS2V5 ³³ was created. For N-terminal tagging a repair donor
377 plasmid consisting of mCherry2 (derived from Addgene plasmid # 72831), flanked by a
378 total of 6X Flag repeat epitope tags was generated. Plasmids for Cas9 and homology
379 repair were transfected to cells with Lipofectamine 2000 (Invitrogen). Successful C-
380 terminal knock-in cells were identified by fluorescence activated cell sorting (FACS) and
381 confirmed by both visual inspection and genomic DNA PCR genotyping, as described ³⁴.
382 A clone with two C-terminal knock-in alleles was transfected with Cas9 and N-terminal
383 homology repair plasmids, followed by FACS to isolate dual mCherry2 and mClover
384 positive edited cells. PCR genotyping revealed that all N-terminal knock-in clones had
385 only one of two alleles successfully incorporating 6xFlag-mCherry2. PCR primers to
386 detect the wild type locus could also discriminate a larger product corresponding to the
387 recombinant allele, however, wild type test reactions were optimized and used only to
388 detect the presence of wild type alleles.

389 *siRNA*

390 Control siRNA oligonucleotides to luciferase and siRNAs cofilin and ADF were as
391 previously described and characterized¹⁵. siRNAs to SRF were from Ambion (#13429)
392 and GCGTGAAGA TCA AGATGGA obtained from Qiagen. Cells were transfected 50 nM
393 of siRNAs with Lipofectamine RNAiMax according to manufacturer's instructions.

394 *smiFISH*

395 Cells were fixed for 20 min at room temp in 4% paraformaldehyde (Electron Microscopy
396 Sciences) in PBS, washed in PBS and permeabilized in 70% ethanol overnight at 4 °C. A
397 set of 30 probes spanning the entire coding sequence of human MYH9 were designed
398 using Oligostan software³⁵ and obtained from IDT. Probes were prepared and annealed
399 according to ref 35, mixed with hybridization buffer (Stellaris) and incubated with cells
400 overnight at 37°C. Cells were washed 3x over 30 min in Wash Buffer A (Stellaris), washed
401 once with Wash Buffer B (Stellaris) and mounted for imaging.

402 *Fluorescence staining*

403 We selected a clone of C-terminally Clover-24xMS2 tagged MYH9 cells that stably
404 expressed Halo-MCP at relatively low levels for visualization of endogenous MYH9
405 mRNAs. Prior to imaging of mRNAs cells were exposed to 200 nM JF646 HaloTag ligand
406 (Promega) for 20 mins. Cells grown on glass coverslips were fixed in 4% formaldehyde
407 in CBS buffer (10 mM 4-Morpholineethanesulfonic acid, pH 6.1, 138 mM KCl, 3 mM
408 MgCl₂, 2 mM ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid, 0.32 M
409 sucrose) for 20 min at room temperature. mRNAs and cytoskeletal structures were best
410 visualized by inclusion of 0.3% Triton X-100 in the fix buffer. This cytoskeletal fixation
411 procedure enhanced myosin-2A labeling in cytoskeletal structures but resulted in less
412 focal labeling of translation sites as detected by anti-Flag. For best quantitative measures
413 of mRNAs and translation sites, replicate coverslips were fixed with and without inclusion
414 of Triton X-100 to visualize mRNAs and translation sites independently. Translation sites
415 in fixed cells were labeled anti-Flag labeling (FUJIFILM, Wako) followed by Cy3 or Alexa
416 594 conjugated anti-mouse secondary antibodies (Jackson Immunoresearch and
417 Invitrogen). In live cells, translation sites were labeled by bead loading Flag-Cy3 Fab

418 antibody fragments as previously described³⁶. Cy3-Flag antibodies detect nascent Flag-
419 MYH9 proteins where both mCherry and mClover signals are absent due to the delay in
420 fluorescence protein maturation. Other antibodies were MKL1 (NBP2-45862, Novus
421 Biologicals; sc-32909 Santa Cruz Biotechnology), SRF (66742-1, Proteintech), GAPDH
422 (MAB 374, Millipore), Sun2 (HPA001209, Sigma). DNA was labeled by 4',6-diamidino-2-
423 phenylindole (DAPI). Mitotic cells were classified based on visual inspection of DAPI
424 labeling.

425 Cells co-transfected with either control or cofilin/ADF siRNAs and an HA-KDM5B-MS2
426 translation reporter⁴ were fixed by the cytoskeletal fixation procedure described above.
427 Cells were labeled for translation by anti-HA antibodies (12CA5, Roche) and with JF646
428 HaloTag ligand for mRNAs.

429 *Microscopy*

430 Confocal images were captured on an Olympus IX8 spinning disk microscope with a
431 CSU22 head, equipped with 405, 488, 561 and 640 nm laser lines. Objectives were either
432 100x/1.40 NA or 60x/1.42 NA. Images were acquired with an iXon Ultra 888 EMCCD
433 camera (Andor) using SlideBook (Intelligent Imaging Innovations). Images of fixed cells
434 were acquired as z-stacks at 0.3 μm intervals.

435 Cells were plated on glass bottom 35 mm dishes in an enclosed chamber at 37°C and
436 with 5% CO₂ for confocal live cell imaging. 11-15 z-planes were acquired at 0.5 μm steps
437 at time intervals between 5 and 10 minutes for evaluations of MYH9 transcription. Live
438 cell translation images were captured as volumes 7-10 z-planes, at 0.65 μm steps and
439 time intervals of 2-10 seconds. Some live cell images were captured on a custom built
440 highly inclined and laminated optical sheet (HILO) microscope⁴ at similar or faster capture
441 rates.

442 *Morphometric measurements*

443 Cell, nuclear and cytoplasmic measurements were obtained from 2D segmentations
444 produced using Cellpose software³⁷. Cell masks were obtained using the 'cyto' model
445 where image channels marking both cells and DAPI labeled nuclei were utilized. Nuclear

446 masks were generated using the ‘nuclei’ model. Integrated intensity measurements were
447 computed using measures over entire z-stacks from background subtracted images,
448 Measurements of myosin stress fiber ordering through filament anisotropy was done
449 using FibrilTool³⁸. A rectangular ROI covering the central portion, over the entire nucleus,
450 of individual cells was applied to images of ventral actomyosin filaments for
451 measurements.

452 *Translation site and mRNA Quantification*

453 A subset of maximum intensity z-projections of control or puromycin treated cells, stained
454 with anti-Flag, were used to annotate translation sites for interactive pixel classification
455 machine learning using Ilastik software³⁹. Visually, translation sites were apparent as
456 bright rounded focal spots. All available features from Color/Intensity, Edge and Texture
457 at scales up to smoothing sigma = 5 were selected. Annotations were made for three
458 classes including, background, translation sites and cell. The trained model was used to
459 generate translation site prediction maps for each experimental dataset, that were then
460 segmented in ImageJ to generate centroid coordinates for each detected site. Intensity
461 measurements were made from a central circular ROI with a radius of 4 pixels, using
462 measurements from a 1-2 pixels ROI extension for localized background subtraction.
463 Where indicated, intensity values were normalized between 0 and 1 for all treatments
464 within a replicate.

465 Images of labeled mRNAs were processed in 3D using FISH-quant v2⁴⁰ for detection
466 and quantification of single mRNAs and nuclear mRNA clusters corresponding to
467 transcription sites. A standard FISH-quant pipeline consisted of application of a Laplacian
468 of Gaussian filter to raw mRNA images, followed by spot detection with parameters: voxel
469 size (nm) in 3D = (300, 95, 95), object radius (nm) = (350, 150, 150) and a threshold
470 scaling value of 1.2. Cluster detection values: radius = 350 nm and minimum # spots = 3.
471 Translation sites and mRNAs were mapped to individual cells and nuclei using
472 segmentations of both structures generated by Cellpose. Code from scripts used for
473 image analysis is available at [https://github.com/Colorado-State-University-Stasevich-](https://github.com/Colorado-State-University-Stasevich-Lab)
474 Lab.

475

476 *Live cell transcription and translation time series analysis*

477 Transcription sites could be identified as bright pulsatile nuclear spots with intensities
478 greater than three times the mean of single nuclear mRNAs. Visually, cells with
479 transcriptional activity could be readily identified from time series projections of nuclear
480 mRNA distribution. Transcription sites were tracked over time using semi-automated
481 tracking with ImageJ TrackMate ⁴¹. Frames in which the tracked spot was absent were
482 assigned the position of the last previous observed spot position. Trajectories of spot
483 intensities measured over a 7-pixel radius from spot centroids were used for classification
484 of ON/OFF periods using a Hidden Markov Model (HMM) as introduced previously ¹⁸. We
485 applied a three-state model in line with an earlier report ⁴². The GaussianHMM function
486 from the python library hmmlearn was used to compute the HMM states for individual
487 transcription site intensity traces.

488 Tracks of translation site motility were generated with TrackMate and mean squared
489 displacement (MSD) measurements computed using opensource TrackPy.

490

491 *Statistical Analysis*

492 Data were tabulated from the results of three to eight experiments, with overlap between
493 datasets for some individual plots such as figures 1E and 5C. P-values were computed
494 using the Welch's t-test.

495

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502

503 **Competing interests**

504 The authors declare no competing interests.

505 **References**

506

- 507 1. Yan, X., Hoek, T.A., Vale, R.D. & Tanenbaum, M.E. Dynamics of Translation of Single
508 mRNA Molecules In Vivo. *Cell* **165**, 976-989 (2016).
- 509 2. Wu, B., Eliscovich, C., Yoon, Y.J. & Singer, R.H. Translation dynamics of single mRNAs in
510 live cells and neurons. *Science* **352**, 1430-1435 (2016).
- 511 3. Wang, C., Han, B., Zhou, R. & Zhuang, X. Real-Time Imaging of Translation on Single
512 mRNA Transcripts in Live Cells. *Cell* **165**, 990-1001 (2016).
- 513 4. Morisaki, T. *et al.* Real-time quantification of single RNA translation dynamics in living cells.
514 *Science* **352**, 1425-1429 (2016).
- 515 5. Morisaki, T., Wiggan, O. & Stasevich, T.J. Translation Dynamics of Single mRNAs in Live
516 Cells. *Annual review of biophysics* (2023).
- 517 6. Buccitelli, C. & Selbach, M. mRNAs, proteins and the emerging principles of gene
518 expression control. *Nature reviews. Genetics* **21**, 630-644 (2020).
- 519 7. Pecci, A., Ma, X., Savoia, A. & Adelstein, R.S. MYH9: Structure, functions and role of non-
520 muscle myosin IIA in human disease. *Gene* **664**, 152-167 (2018).
- 521 8. Eisenberg, E. & Levanon, E.Y. Human housekeeping genes, revisited. *Trends Genet* **29**,
522 569-574 (2013).
- 523 9. Amano, M. *et al.* Phosphorylation and activation of myosin by Rho-associated kinase
524 (Rho-kinase). *J.Biol.Chem.* **271**, 20246-20249 (1996).
- 525 10. Nguyen, L.T.S., Jacob, M.A.C., Parajón, E. & Robinson, D.N. Cancer as a biophysical
526 disease: Targeting the mechanical-adaptability program. *Biophysical journal* **121**, 3573-
527 3585 (2022).
- 528 11. Wang, Y., Liu, S., Zhang, Y. & Yang, J. Myosin Heavy Chain 9: Oncogene or Tumor
529 Suppressor Gene? *Medical science monitor : international medical journal of experimental
530 and clinical research* **25**, 888-892 (2019).
- 531 12. Medjkane, S., Perez-Sanchez, C., Gaggioli, C., Sahai, E. & Treisman, R. Myocardin-
532 related transcription factors and SRF are required for cytoskeletal dynamics and
533 experimental metastasis. *Nat Cell Biol* **11**, 257-268 (2009).
- 534 13. Pichon, X. *et al.* Visualization of single endogenous polysomes reveals the dynamics of
535 translation in live human cells. *J Cell Biol* **214**, 769-781 (2016).
- 536 14. Stonyte, V., Boye, E. & Grallert, B. Regulation of global translation during the cell cycle. *J
537 Cell Sci* **131** (2018).
- 538 15. Wiggan, O., Shaw, A.E., DeLuca, J.G. & Bamburg, J.R. ADF/cofilin regulates actomyosin
539 assembly through competitive inhibition of myosin II binding to F-actin. *Developmental
540 Cell* **22**, 530-543 (2012).
- 541 16. Wiggan, O., Schroder, B., Krapf, D., Bamburg, J.R. & DeLuca, J.G. Cofilin Regulates
542 Nuclear Architecture through a Myosin-II Dependent Mechanotransduction Module.
543 *Scientific reports* **7**, 40953 (2017).
- 544 17. Verdoni, A.M., Aoyama, N., Ikeda, A. & Ikeda, S. Effect of destrin mutations on the gene
545 expression profile in vivo. *Physiological genomics* **34**, 9-21 (2008).
- 546 18. Larson, D.R. *et al.* Direct observation of frequency modulated transcription in single cells
547 using light activation. *eLife* **2**, e00750 (2013).
- 548 19. Tunnacliffe, E. & Chubb, J.R. What Is a Transcriptional Burst? *Trends Genet* **36**, 288-297
549 (2020).
- 550 20. Rodriguez, J. *et al.* Intrinsic Dynamics of a Human Gene Reveal the Basis of Expression
551 Heterogeneity. *Cell* **176**, 213-226.e218 (2019).
- 552 21. Dar, R.D. *et al.* Transcriptional burst frequency and burst size are equally modulated
553 across the human genome. *Proc Natl Acad Sci U S A* **109**, 17454-17459 (2012).

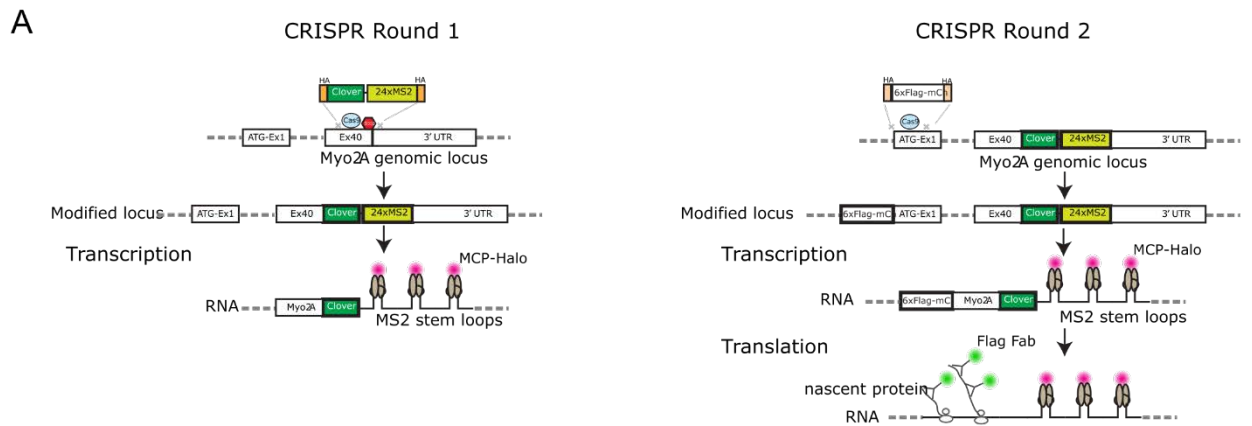
- 554 22. Kalo, A. *et al.* Cellular Levels of Signaling Factors Are Sensed by β -actin Alleles to
555 Modulate Transcriptional Pulse Intensity. *Cell reports* **11**, 419-432 (2015).
- 556 23. Olson, E.N. & Nordheim, A. Linking actin dynamics and gene transcription to drive cellular
557 motile functions. *Nature reviews. Molecular cell biology* **11**, 353-365 (2010).
- 558 24. Schofield, A. & Bernard, O. LIM Kinase and Cancer Metastasis, in *Cytoskeleton and*
559 *Human Disease*. (ed. M. Kavallaris) 159-168 (Humana Press, Totowa, NJ; 2012).
- 560 25. Miralles, F., Posern, G., Zaromytidou, A.I. & Treisman, R. Actin dynamics control SRF
561 activity by regulation of its coactivator MAL. *Cell* **113**, 329-342 (2003).
- 562 26. Ridley, A.J. & Hall, A. The small GTP-binding protein rho regulates the assembly of focal
563 adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399 (1992).
- 564 27. Hill, C.S., Wynne, J. & Treisman, R. The Rho family GTPases RhoA, Rac1, and CDC42Hs
565 regulate transcriptional activation by SRF. *Cell* **81**, 1159-1170 (1995).
- 566 28. Chrzanowska-Wodnicka, M. & Burridge, K. Rho-stimulated contractility drives the
567 formation of stress fibers and focal adhesions. *J. Cell Biol.* **133**, 1403-1415 (1996).
- 568 29. Esnault, C. *et al.* Rho-actin signaling to the MRTF coactivators dominates the immediate
569 transcriptional response to serum in fibroblasts. *Genes Dev* **28**, 943-958 (2014).
- 570 30. Yoon, Y.J. *et al.* Glutamate-induced RNA localization and translation in neurons. *Proc Natl*
571 *Acad Sci U S A* **113**, E6877-e6886 (2016).
- 572 31. Takaki, T. *et al.* Actomyosin drives cancer cell nuclear dysmorphia and threatens genome
573 stability. *Nature communications* **8**, 16013 (2017).
- 574 32. Wiggan, O., DeLuca, J.G., Stasevich, T.J. & Bamburg, J.R. Lamin A/C deficiency enables
575 increased myosin-II bipolar filament ensembles that promote divergent actomyosin
576 network anomalies through self-organization. *Mol Biol Cell* **31**, 2363-2378 (2020).
- 577 33. Wu, B. *et al.* Synonymous modification results in high-fidelity gene expression of repetitive
578 protein and nucleotide sequences. *Genes Dev* **29**, 876-886 (2015).
- 579 34. Bauer, D.E., Canver, M.C. & Orkin, S.H. Generation of genomic deletions in mammalian
580 cell lines via CRISPR/Cas9. *Journal of visualized experiments : JoVE*, e52118 (2015).
- 581 35. Tsanov, N. *et al.* smiFISH and FISH-quant - a flexible single RNA detection approach with
582 super-resolution capability. *Nucleic Acids Res* **44**, e165 (2016).
- 583 36. Cialek, C.A., Galindo, G., Koch, A.L., Saxton, M.N. & Stasevich, T.J. Bead Loading
584 Proteins and Nucleic Acids into Adherent Human Cells. *Journal of visualized experiments*
585 *: JoVE* (2021).
- 586 37. Stringer, C., Wang, T., Michaelos, M. & Pachitariu, M. Cellpose: a generalist algorithm for
587 cellular segmentation. *Nature methods* **18**, 100-106 (2021).
- 588 38. Boudaoud, A. *et al.* FibrilTool, an ImageJ plug-in to quantify fibrillar structures in raw
589 microscopy images. *Nature protocols* **9**, 457-463 (2014).
- 590 39. Berg, S. *et al.* ilastik: interactive machine learning for (bio)image analysis. *Nature methods*
591 **16**, 1226-1232 (2019).
- 592 40. Imbert, A. *et al.* FISH-quant v2: a scalable and modular tool for smFISH image analysis.
593 *RNA (New York, N. Y.)* **28**, 786-795 (2022).
- 594 41. Tinevez, J.Y. *et al.* TrackMate: An open and extensible platform for single-particle tracking.
595 *Methods (San Diego, Calif.)* **115**, 80-90 (2017).
- 596 42. Senecal, A. *et al.* Transcription factors modulate c-Fos transcriptional bursts. *Cell reports*
597 **8**, 75-83 (2014).

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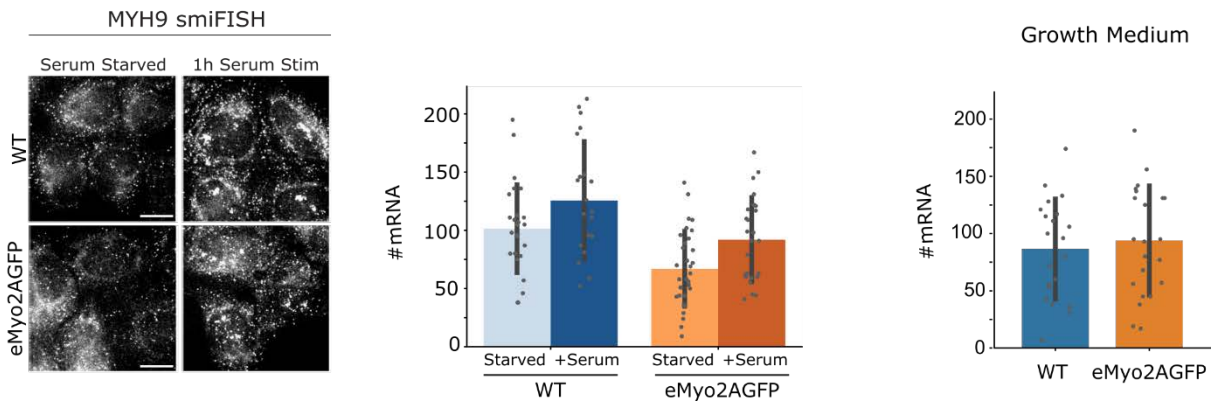
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600 **Supplementary Material**

601



B



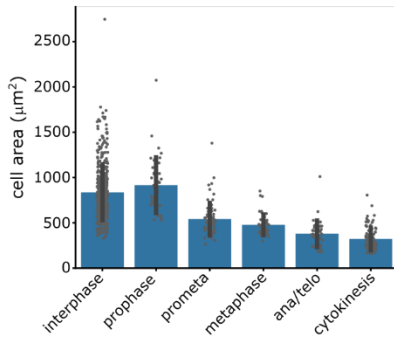
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603 **Figure S1. CRISPR strategy to tag endogenous MYH9 and smiFISH analysis of**
604 **tagged cells. A.** Illustration of sequential gene editing strategy used to generate eMyo2A
605 GFP cells. **B.** Fluorescence images and quantification of MYH9 mRNA labeled by
606 smiFISH probes that target both tagged and untagged transcripts. mRNAs were assessed
607 under normal growth conditions and following serum stimulation of serum starved cells.
608 Values are mean \pm SD. Scale bars 10 μ m.

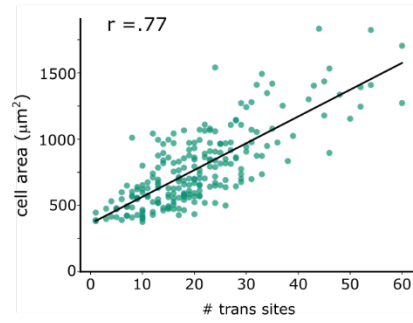
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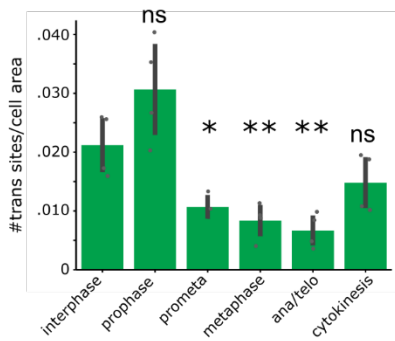
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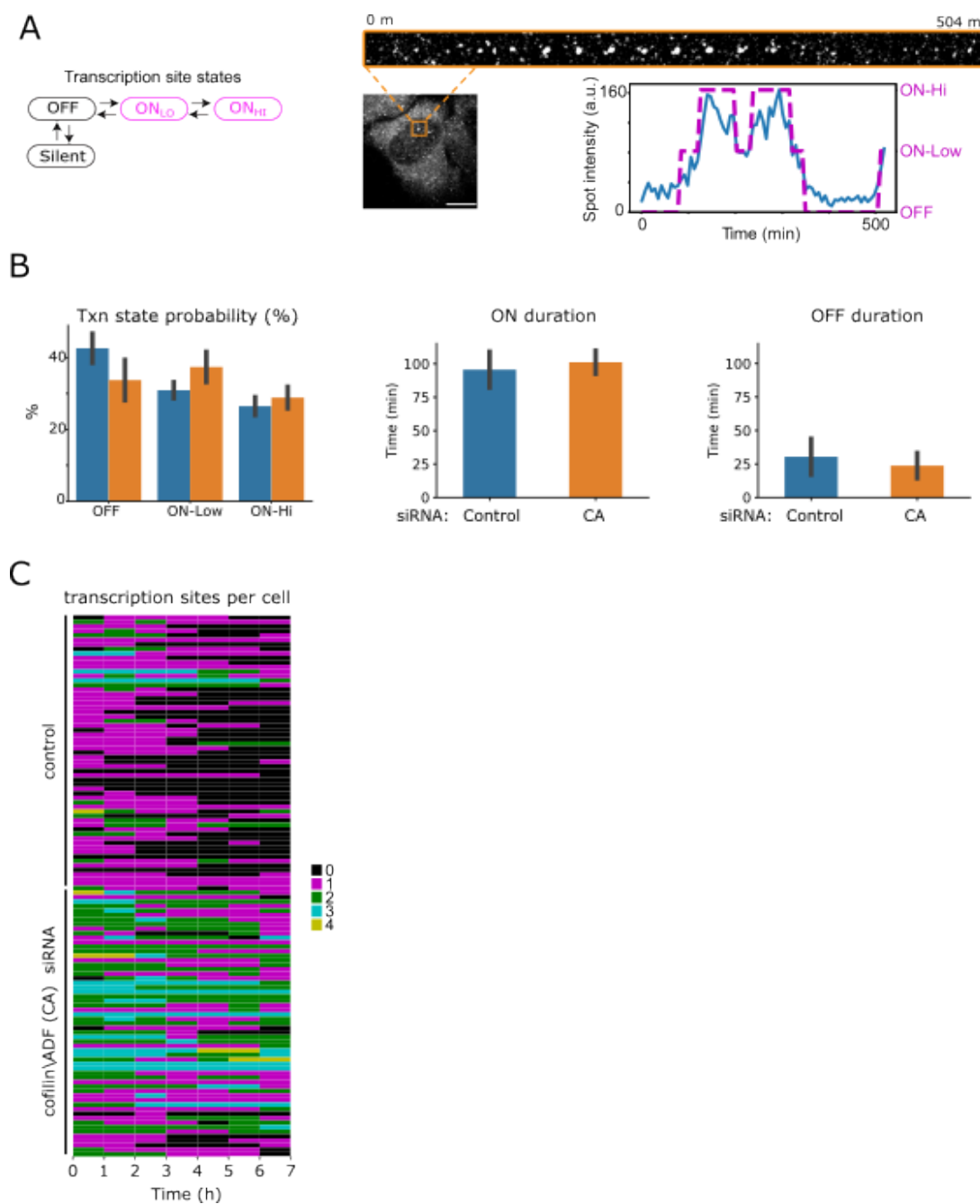
613 **Figure S2. Relationships between MYH9 translation and cell area.** **A.** Quantification
614 of eMyo2AGFP surface cell area across the cell cycle, mean \pm SD. **B.** Correlation plot of
615 cell area to numbers of MYH9 translation sites, r is Pearson's correlation coefficient. **C.**
616 Quantification of MYH9 translation sites normalized to cell area across the cell cycle,
617 mean \pm SD. * $p < 0.05$, ** $p \leq 0.01$, n.s., not significant, Welch's t-test.

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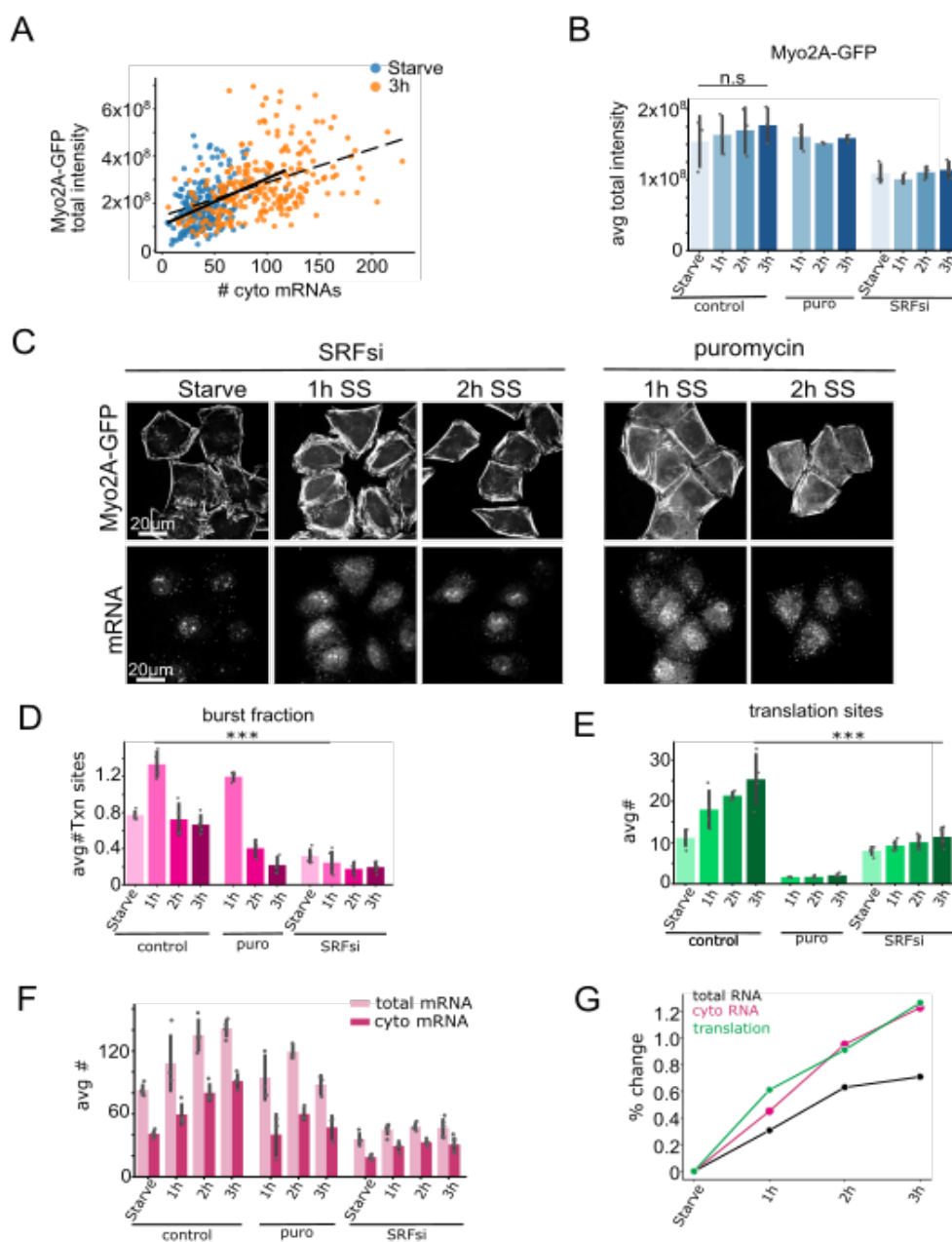
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622

623 **Figure S3. Measurements of MYH9 transcriptional activity in live cells. A.**
 624 Hypothetical transcription activity model and example confocal fluorescence images of
 625 Halo-MCP labeled MYH9 transcription foci (boxed region) in a time-lapse series. Scale
 626 bar 10 μ m. Transcription site intensity trace illustrates classification of on and off states
 627 as fit to a hidden Markov model. **B.** Quantification of MYH9 transcription activity dynamics
 628 for control or cofilin/ADF siRNA treated eMyo2AGFP cells. Values are mean \pm SD. **C.**
 629 Heat map of the number of per cell MYH9 transcription sites over time, with each row
 630 corresponding to an individual cell.



631 **Figure S4. Transcription and translation requirements in MYH9 expression for**
 632 **cytoskeletal response to serum activation.** **A.** Correlation plot of integrated Myosin2A-
 633 GFP intensity to cytoplasmic MYH9 mRNAs for eMyo2AGFP cells. Solid line shows linear
 634 fit to starved condition and dashed line to 3h post serum stimulation. **B.** Quantification of
 635 total Myosin2A-GFP intensity. **C.** Confocal fluorescence images depicting actomyosin
 636 cytoskeletal rearrangements and MYH9 mRNAs following serum stimulation (SS). **D.**
 637 Quantification of the number of per cell MYH9 transcription sites. **E.** Quantification of per
 638 cell translation sites. **F.** Quantification of MYH9 mRNAs. **G.** Percentage change plot
 639 summarizing data for control treatment from panels E and F. Bar graphs are mean \pm SD.
 640 *** $p \leq 0.001$, n.s., not significant, Welch's t-test.

641 Supplementary Movies

642 **Movie S1.** Live cell imaging of an eMyo2AGFP cell with myo2A-GFP protein (green) and
643 mRNA (JF646-Halo-MCP, magenta), boxed region illustrates mRNA transcription focus.
644 Images acquired at 1 frame/second. Related to Fig. 1.

645 **Movie S2.** Live cell imaging of translation from a single mRNA (cyan boxed region,
646 enlarged in spot1) and a mRNA cluster (orange boxed region, enlarged in spot2) in HeLa
647 eMyo2AGFP cells. Translation (trans) and myosin2A protein are labeled by bead loaded
648 Cy3-Flag Fab (green) and mRNAs by JF646-Halo-MCP (magenta). Images are confocal
649 z-stack projections acquired at 0.16 frame (z-volume)/second. Related to Fig. 1.

650 **Movie S3.** Live cell imaging of a MYH9 transcription activity in an eMyo2AGFP cell with
651 mRNA labeled by JF646-Halo-MCP. Images are confocal z-stack projections acquired at
652 7 min intervals. Related to Fig. S3