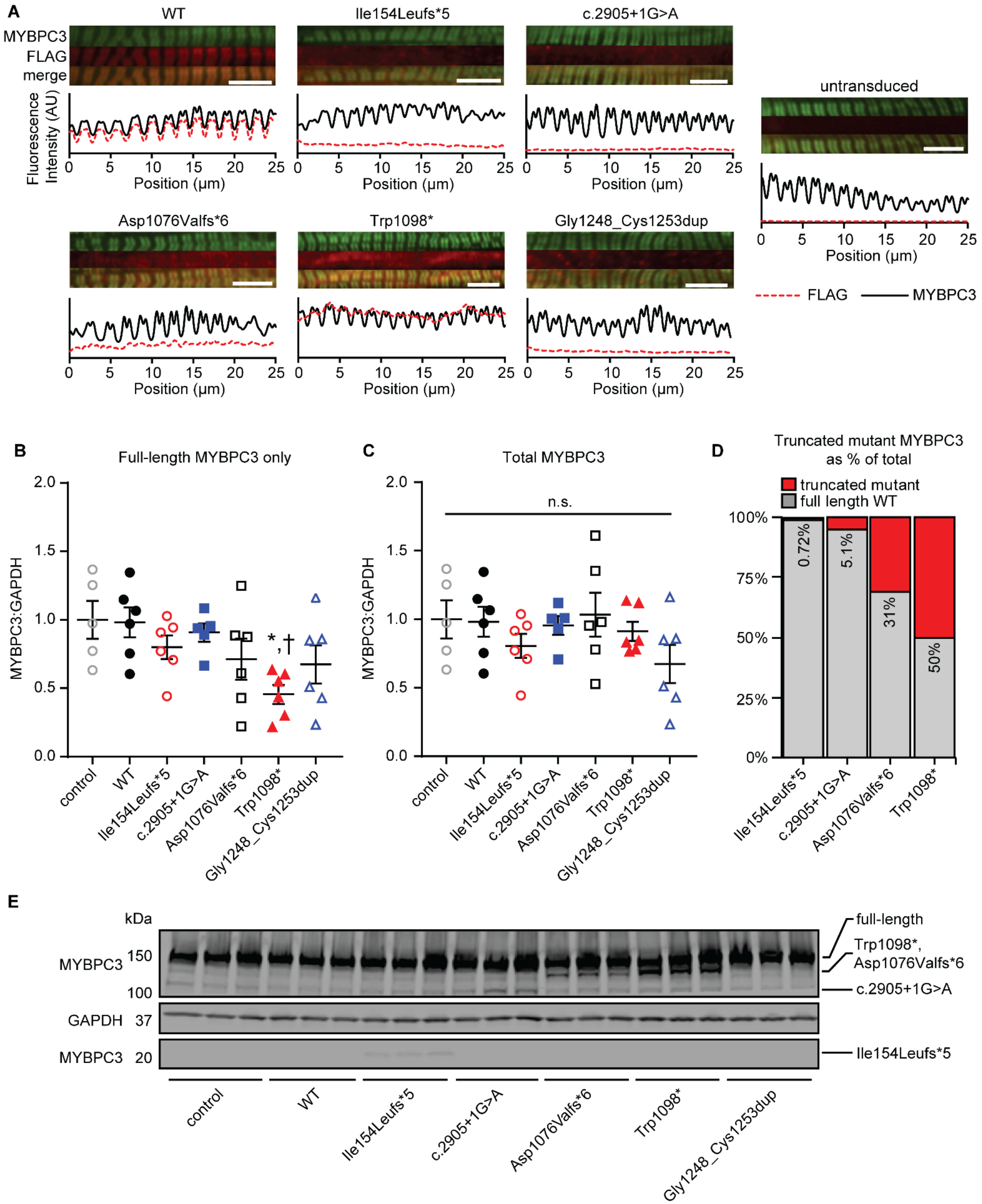
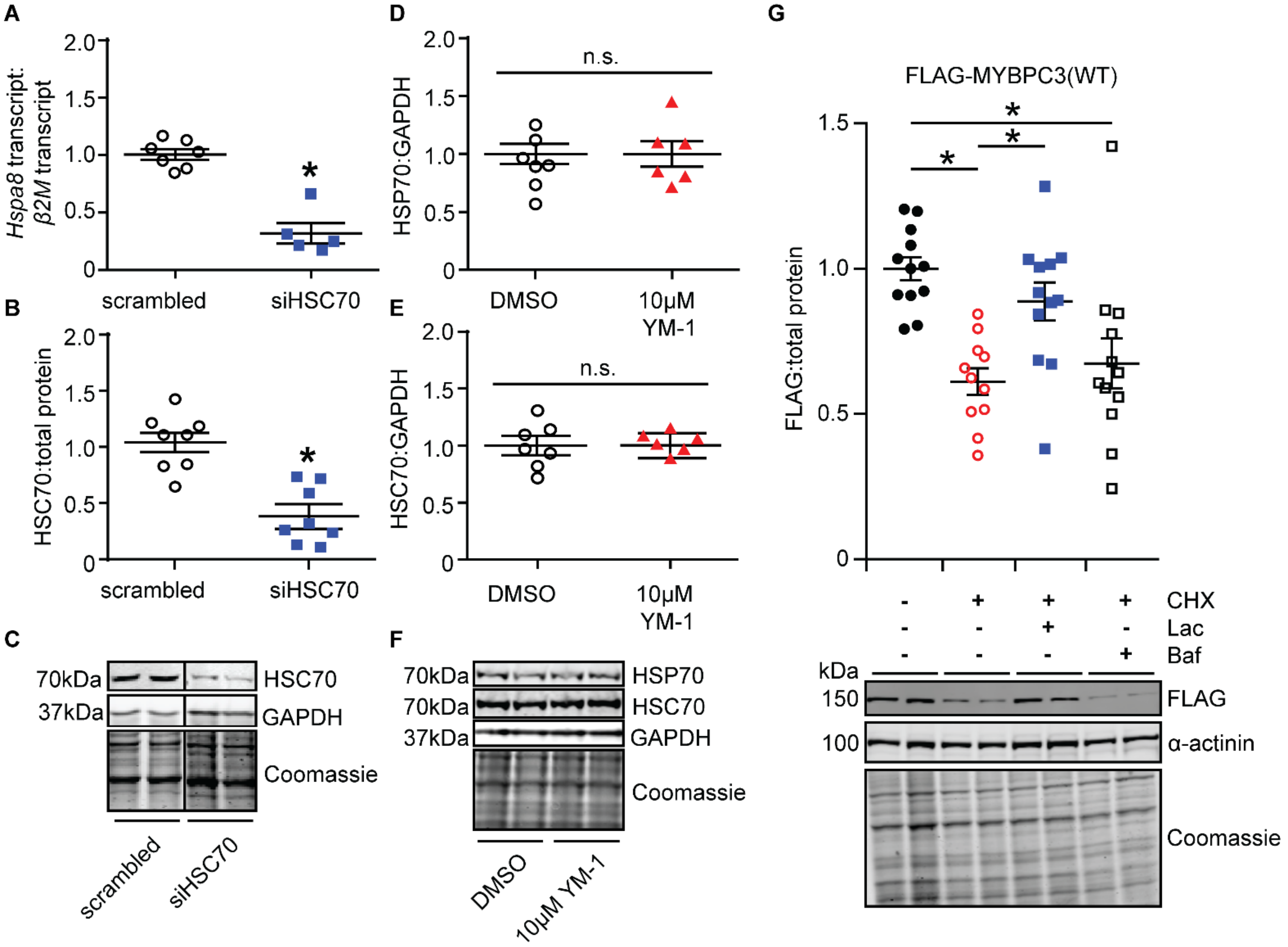

A
WT
Ile154Leufs*5
c.2905+1G>A
MYBPC3
FLAG
merge
Fluorescence Intensity (AU)
0 5 10 15 20 25
Position (µm)
untransduced
Asp1076Valfs*6
Trp1098*
Gly1248_Cys1253dup
FLAG
MYBPC3
B
Full-length MYBPC3 only
MYBPC3:GAPDH
2.0
1.5
1.0
0.5
0.0
*,†
control
WT
Ile154Leufs*5
c.2905+1G>A
Asp1076Valfs*6
Trp1098*
Gly1248_Cys1253dup
C
Total MYBPC3
n.s.
D
Truncated mutant MYBPC3 as % of total
truncated mutant
full length WT
100%
75%
50%
25%
0%
0.72%
5.1%
31%
50%
E
kDa
MYBPC3
150
100
GAPDH
37
MYBPC3
20
full-length
Trp1098*, Asp1076Valfs*6
c.2905+1G>A
Ile154Leufs*5

Supplemental Fig. 1. *(A)* Fluorescence micrographs and corresponding intensity traces from patterned NRVMs treated with FLAG-MYBPC3 adenovirus at MOI 2 for endogenous MYBPC3 (green) and FLAG (red). FLAG-WT properly colocalizes to the C-zone of the sarcomere with endogenous MYBPC3, while FLAG-mutants show no sarcomere periodicity. Scale bar = 20μm. *(B)* Expression of full-length MYBPC3 protein in NRVMs treated with FLAG-MYBPC3 adenovirus. FLAG-WT expression does not change total MYBPC3 expression. Note that FLAG-WT and FLAG-Gly1248_Cys1253dup were not sufficiently separated from endogenous MYBPC3 by SDS-PAGE, and therefore quantification for these conditions includes both endogenous and FLAG-MYBPC3. Expression of truncated FLAG-MYBPC3 tended to reduce endogenous MYBPC3 expression. Mean±SEM, Kruskal Wallis one-way ANOVA $p=0.0282$, $^*p<0.05$, Dunn's test for multiple comparisons, $n\geq5$. *(C)* When both endogenous and FLAG-MYBPC3 signals were combined, no significant differences in total MYBPC3 expression were observed, indicating no overexpression. Mean±SEM, Kruskal Wallis one-way ANOVA $p=0.565$, $n\geq5$. *(D)* Truncated FLAG-MYBPC3 mutant expression as percent of total MYBPC3. *(E)* Representative Western blot for MYBPC3.

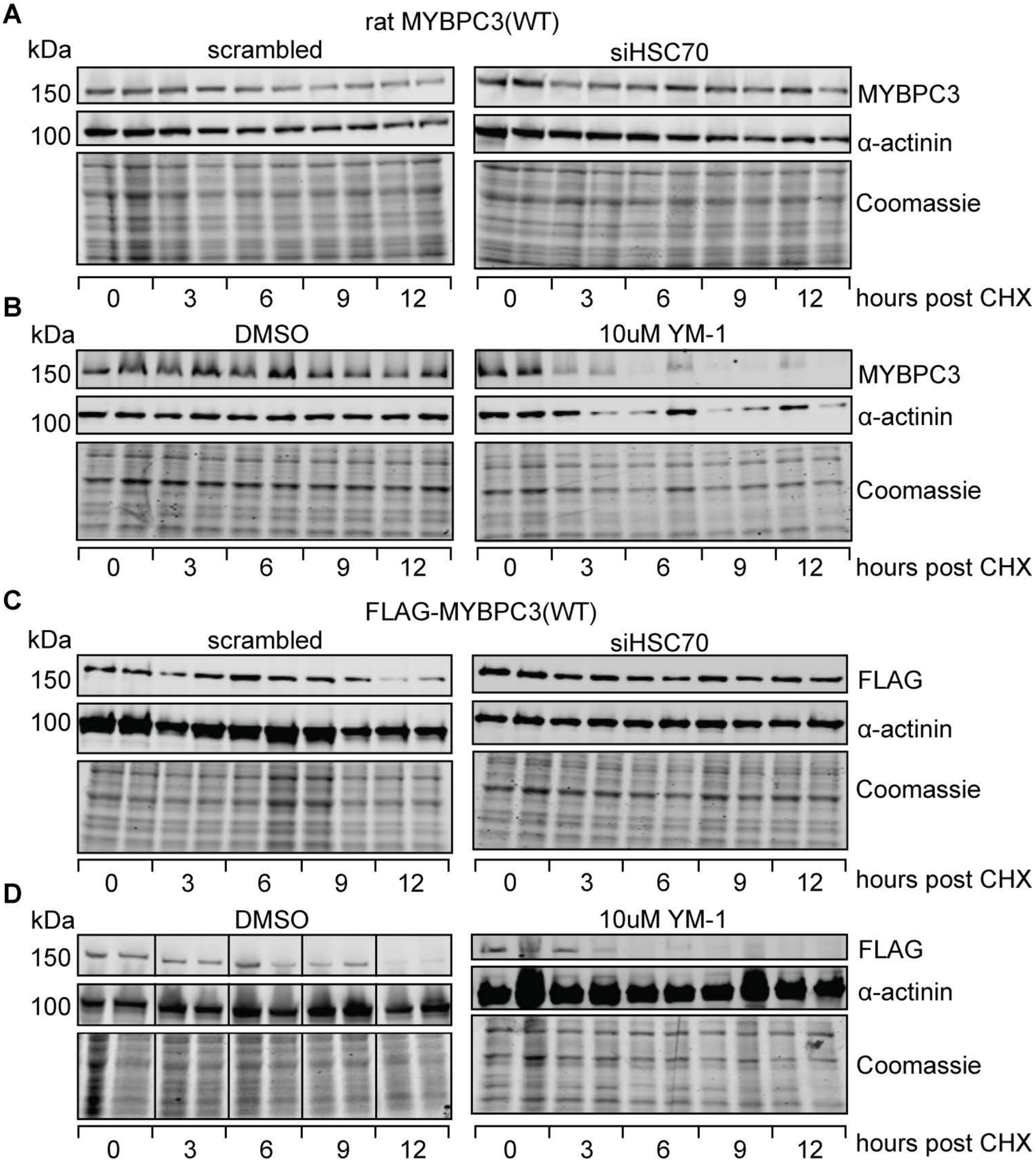
Supplemental Table 1. Overlap in matching peptides for bait vs. endogenous MYBPC3.

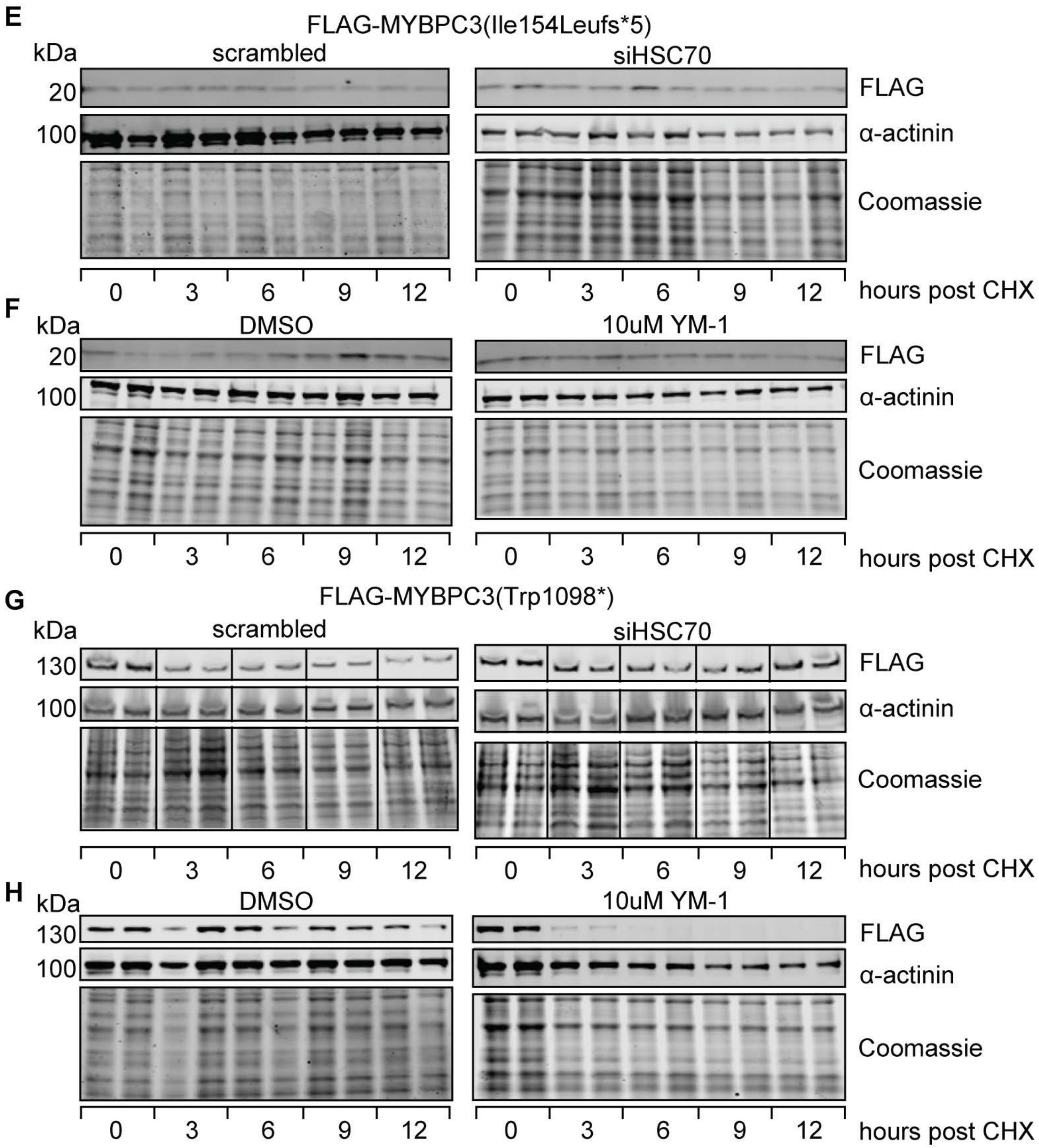
IP-MS Set 1	Peptides unique to bait MYBPC3	common peptides	Peptides unique to Endogenous MYBPC3	Total peptides
Control	86	57	0	143
WT	1709	949	24	2682
Ile154Leufs*5	409	0	58	467
Trp1098*	1350	808	17	2175

IP-MS Set 2	Peptides unique to bait MYBPC3	common peptides	Peptides unique to Endogenous MYBPC3	Total peptides
Control	38	41	3	82
WT	333	263	9	605
Ile154Leufs*5	85	0	52	137
Trp1098*	188	146	20	354



Supplemental Fig. 2. *(A)* NRVMs treated with 20µM siRNA directed against the rat *Hspa8* gene for 24hr show reduced HSC70 transcript compared to treatment with 20µM non-specific scrambled siRNA. (*B)* HSC70 protein is also reduced 24hr after siRNA treatment. Mean±SEM, *p<0.05 vs scrambled, student's t test. *(C)* Representative Western blot and total protein from siRNA-treated cells. *(D and E)* YM-1 treatment does not affect protein levels of HSP70 or HSC70, respectively*.* Mean±SEM*. (F)* Representative Western blot and total protein from YM-1 and DMSO vehicle-treated cells. *(G)* FLAG-MYBPC3(WT) is primarily degraded through the proteasome rather than autophagy. Quantification of MYBPC3 and representative Western blot from NRVMs treated with 300µg/mL CHX for 12 hr. Addition of 25µM of the proteasome inhibitor lactacystin (Lac) along with CHX prevented degradation of MYBPC3, while addition of 50nM of the autophagy inhibitor bafilomycin (Baf) along with CHX did not. Kruskal-Wallis one-way ANOVA p=0.0001, *p<0.05 Dunn's test for multiple comparisons,. Mean±SEM. n=12. Black line between lanes indicates noncontiguous samples from the same blot.


A
rat MYBPC3(WT)
kDa
scrambled
siHSC70
150
100
MYBPC3
α-actinin
Coomassie
0
3
6
9
12
hours post CHX
B
kDa
DMSO
10uM YM-1
150
100
MYBPC3
α-actinin
Coomassie
0
3
6
9
12
hours post CHX
C
FLAG-MYBPC3(WT)
kDa
scrambled
siHSC70
150
100
FLAG
α-actinin
Coomassie
0
3
6
9
12
hours post CHX
D
kDa
DMSO
10uM YM-1
150
100
FLAG
α-actinin
Coomassie
0
3
6
9
12
hours post CHX


E
FLAG-MYBPC3(Ile154Leufs*5)
kDa
scrambled
siHSC70
20
100
FLAG
α-actinin
Coomassie
0
3
6
9
12
hours post CHX
F
kDa
DMSO
10uM YM-1
20
100
FLAG
α-actinin
Coomassie
0
3
6
9
12
hours post CHX
G
FLAG-MYBPC3(Trp1098*)
kDa
scrambled
siHSC70
130
100
FLAG
α-actinin
Coomassie
0
3
6
9
12
hours post CHX
H
kDa
DMSO
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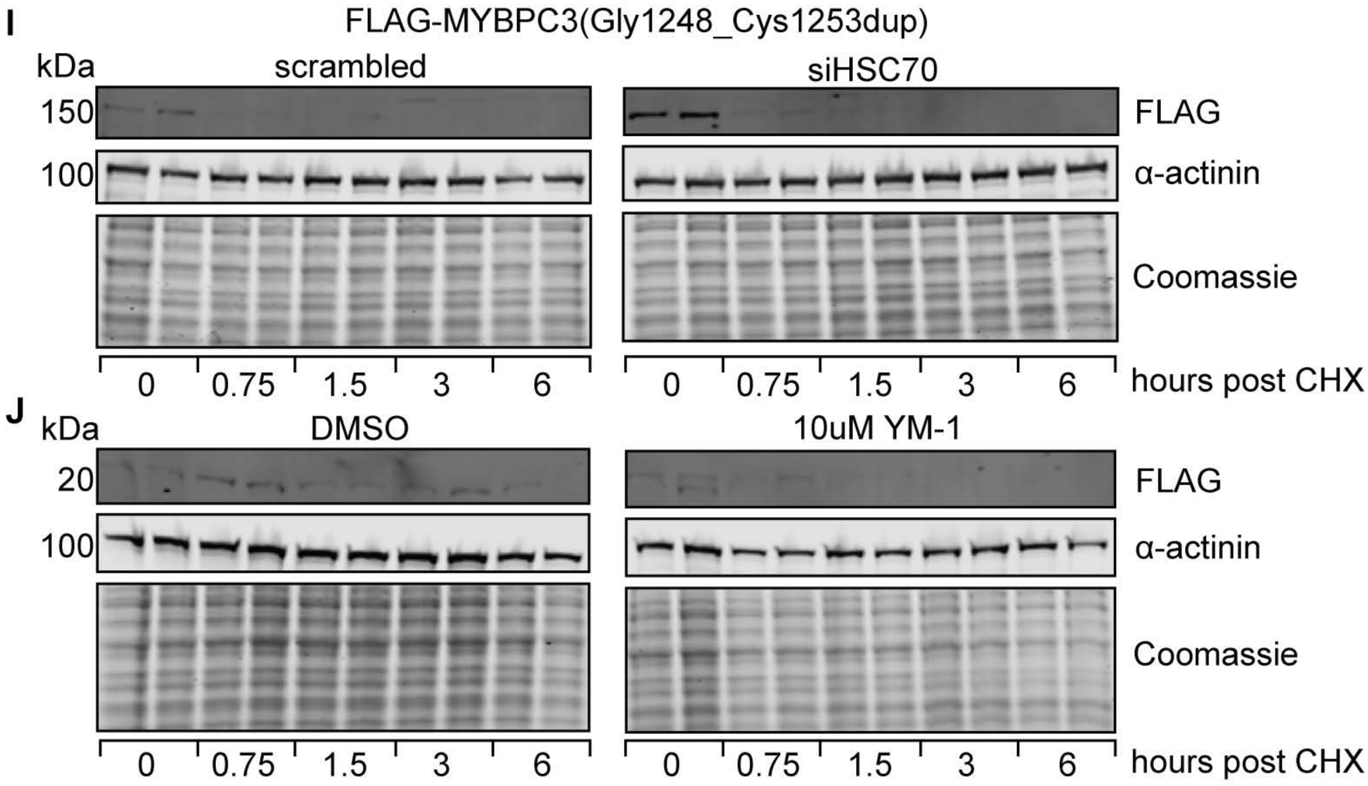
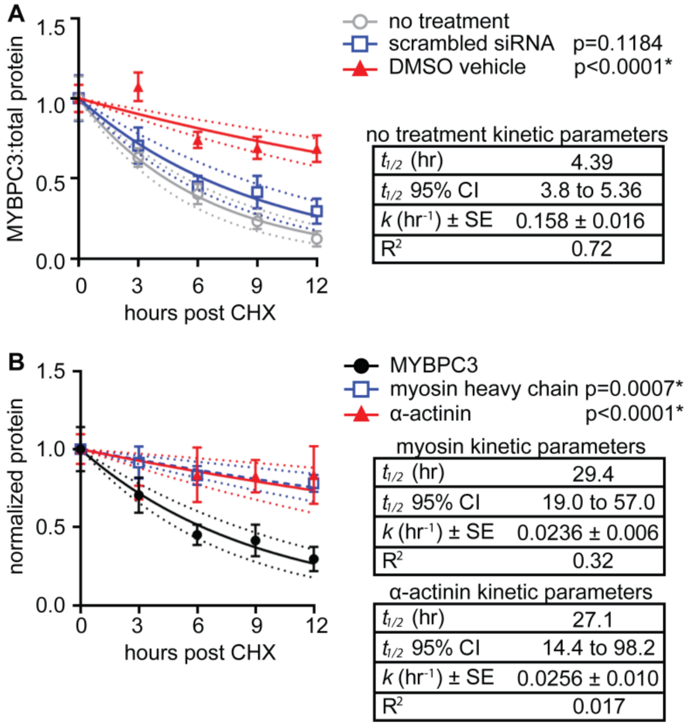
130
100
FLAG
α-actinin
Coomassie
0
3
6
9
12
hours post CHX



Supplemental Figure 3. Representative Western blots for MYBPC3 cycloheximide experiments. Blots are representative of three independent experiments. Black line between lanes indicates noncontiguous samples from the same blot. Lanes omitted were additional replicates of conditions shown. *(A,B)* Endogenous rat WT MYBPC3, *(C,D)* FLAG-WT MYBPC3, *(E,F)* FLAG-Ile154Leufs*5 MYBPC3, *(G,H)* FLAG-Trp1098* MYBPC3, *(I,J)* FLAG-Gly1248_Cys1253dup MYBPC3.



no treatment kinetic parameters

$t_{1/2}$ (hr)	4.39
$t_{1/2}$ 95% CI	3.8 to 5.36
k (hr^{-1}) ± SE	0.158 ± 0.016
R^2	0.72

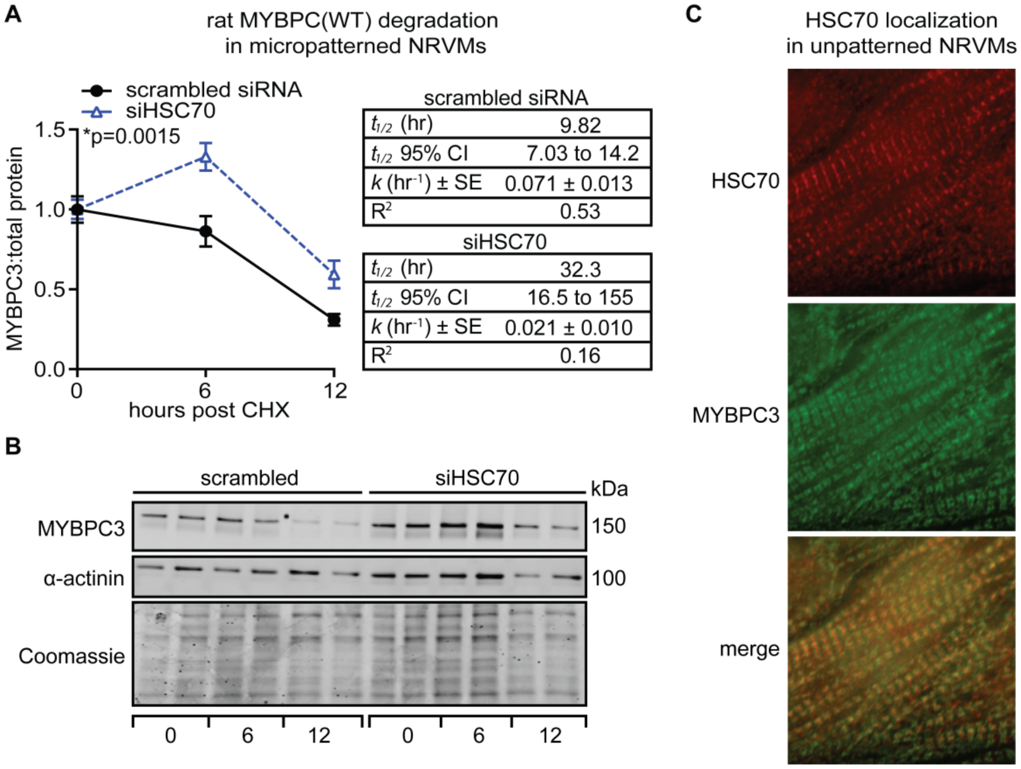
myosin kinetic parameters

$t_{1/2}$ (hr)	29.4
$t_{1/2}$ 95% CI	19.0 to 57.0
k (hr^{-1}) ± SE	0.0236 ± 0.006
R^2	0.32

α-actinin kinetic parameters

$t_{1/2}$ (hr)	27.1
$t_{1/2}$ 95% CI	14.4 to 98.2
k (hr^{-1}) ± SE	0.0256 ± 0.010
R^2	0.017

Supplemental Fig. 4. *(A)* Comparison of endogenous rat MYBPC3 degradation rates between no treatment, scrambled siRNA, and DMSO vehicle. Scrambled siRNA did not significantly affect degradation of MYBPC3, while DMSO significantly slowed degradation. Mean±SEM, n=4 for no treatment. Scrambled and DMSO data were previously shown in Figure 3 and Table 1 of the main text. *(B)* Degradation of myosin heavy chain and α-actinin with scrambled siRNA treatment. Rate constants *k* for degradation of myosin and α-actinin are significantly different compared to endogenous MYBPC3 under the same conditions. Mean±SEM, n=4 for myosin, n≥7 for α-actinin. Dotted lines indicate 95% confidence intervals for curve fits.



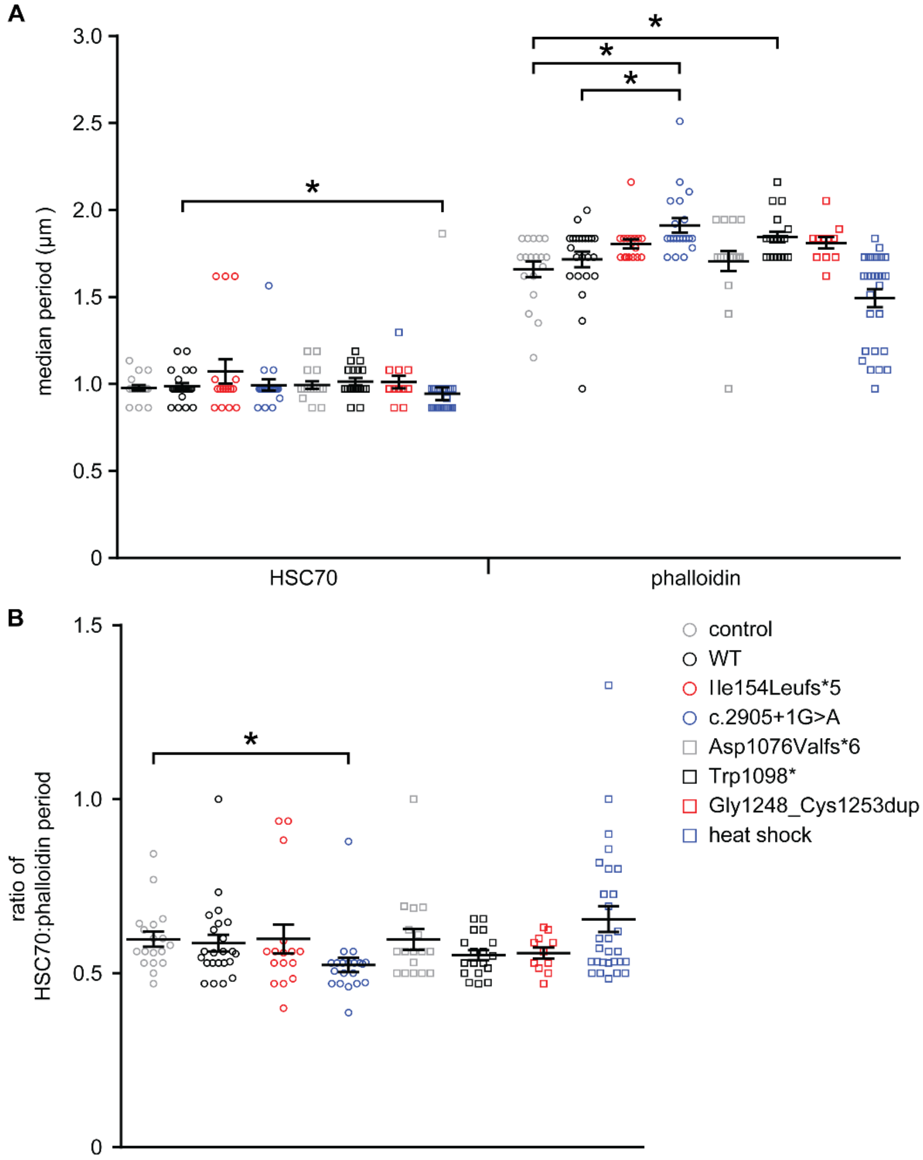
scrambled siRNA

$t_{1/2}$ (hr)	9.82
$t_{1/2}$ 95% CI	7.03 to 14.2
k (hr^{-1}) ± SE	0.071 ± 0.013
R^2	0.53

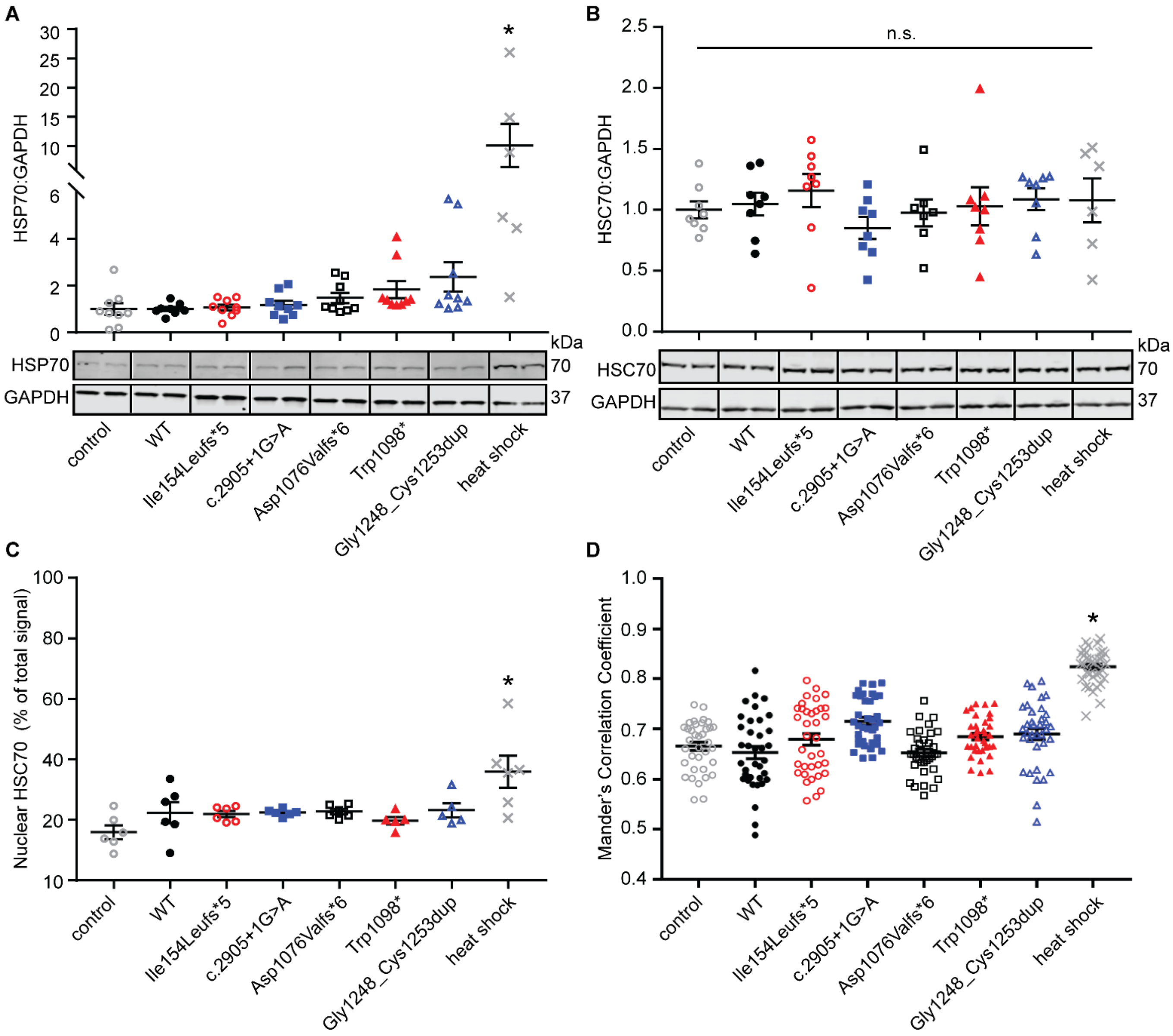
siHSC70

$t_{1/2}$ (hr)	32.3
$t_{1/2}$ 95% CI	16.5 to 155
k (hr^{-1}) ± SE	0.021 ± 0.010
R^2	0.16

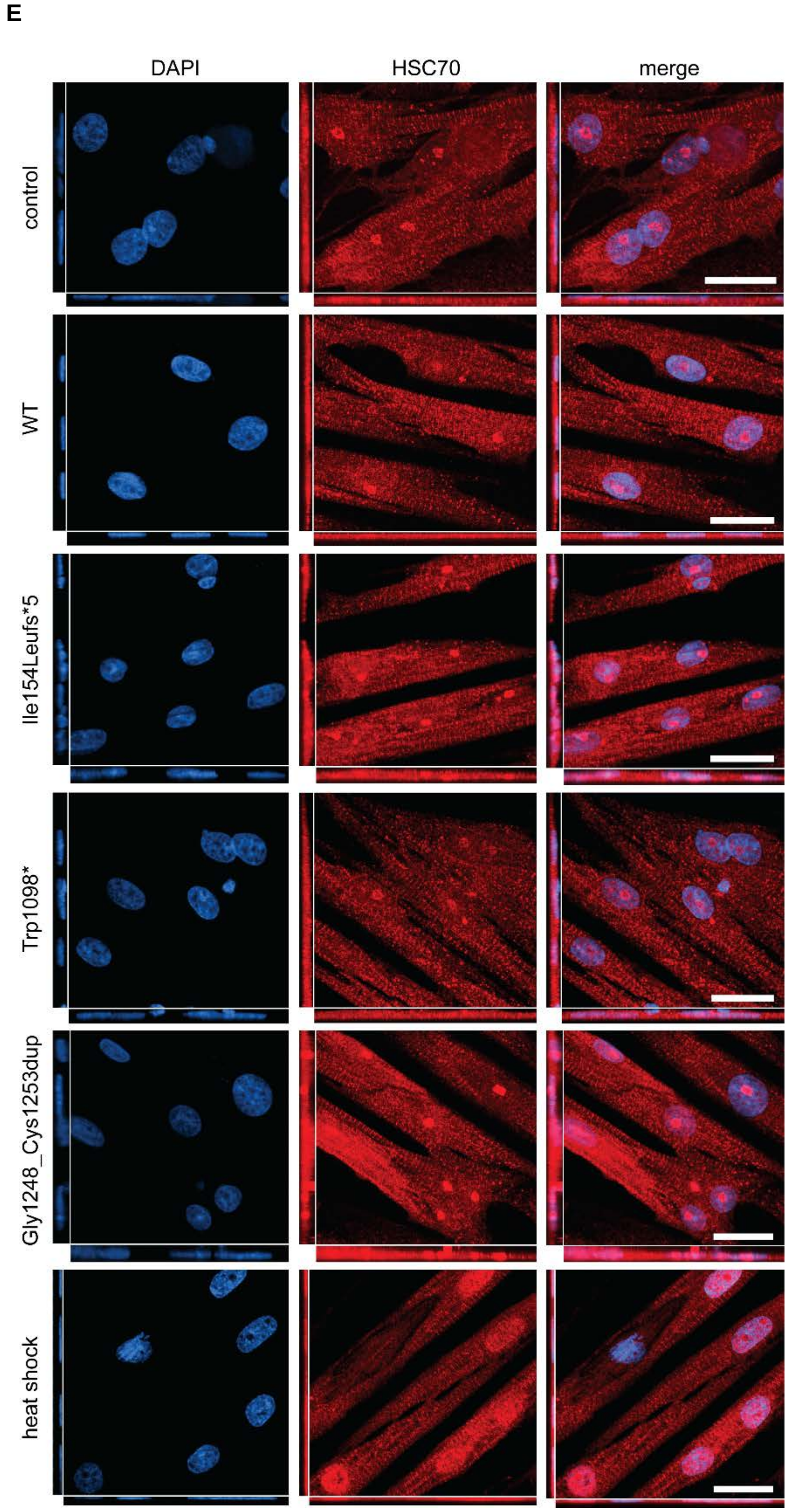
Supplemental Figure 5. *(A)* Degradation of endogenous rat MYBPC3 in micropatterned NRVMs. Micropatterning affected the $t_{½}$ of MYBPC3, but did not influence the effect of HSC70 knockdown. Mean±SEM, n≥9. *(B)* Representative Western blot from cycloheximide assay performed in micropatterned NRVMs. *(C)* Immunostaining for HSC70 and MYBPC3 in unpatterned NRVMs. HSC70 localizes to the M-line and Z-disk in both micropatterned cells and unpatterned monolayers. 60X magnification.



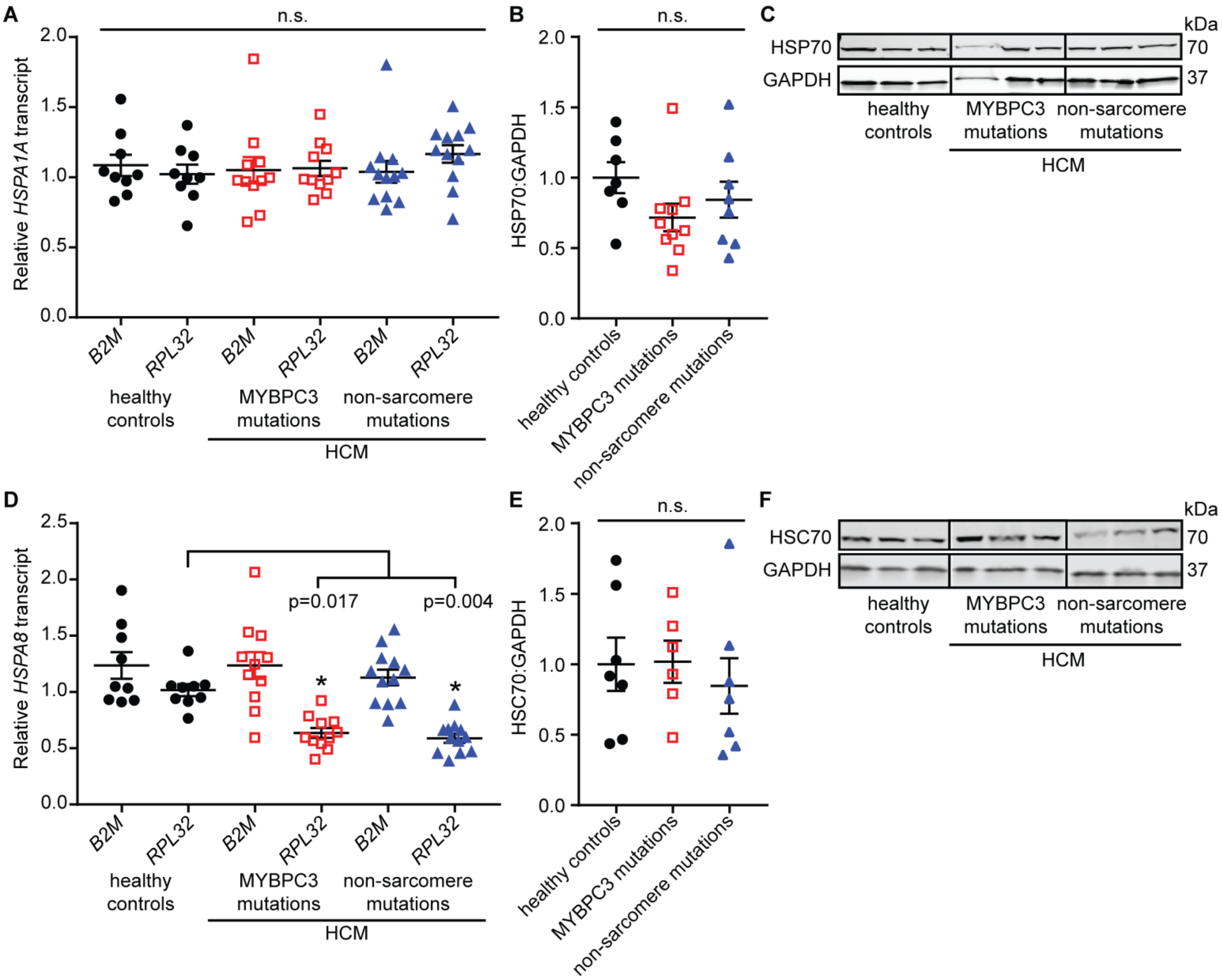
Supplemental Fig 6. Mutant MYBPC3 protein expression does not alter HSC70 sarcomere periodicity. *(A)* Frequency of HSC70 and phalloidin peaks in untreated control vs. FLAG-MYBPC3 adenovirus treated and heat shocked NRVMs, determined by fast Fourier transform analyses on fluorescent intensity profiles. HSC70 frequency was not significantly affected by mutant MYBPC3 protein expression or heat shock. *(B)* Only cells expressing the c.2905+1G>A mutant showed a significantly altered ratio of HSC70 frequency to phalloidin frequency when compared to untreated cells, but not FLAG-WT treated cells. Kruskal-Wallis one-way ANOVA with Dunn’s test for multiple comparisons, *$p<0.05$. Mean±SEM. n≥16 cells per condition.



Supplemental Fig. 7. Mutant MYBPC3 expression in NRVMs does not induce HSP70 upregulation or HSC70 nuclear translocation. *(A)* Quantification and representative Western blot for HSP70 (stress-inducible). HSP70 protein levels were only significantly elevated in NRVMs heat shocked for 1 hour at 45°C. n≥6, Kruskal-Wallis one way ANOVA p=0.003. *p<0.05 vs control, Dunn's test for multiple comparisons. Mean±SEM. *(B)* Quantification and representative Western blot for HSC70. HSC70 protein levels were consistent across all conditions. n≥6, Kruskal-Wallis one-way ANOVA p=0.48. Mean±SEM. *(C)* Immunofluorescence-based Quantification of HSC70 signal localized to DAPI-positive areas as a percent of total signal. Only heat shocked cells showed significantly increased nuclear localization of HSC70. n≥5 fields of view, Kruskal-Wallis one way ANOVA p=0.021. *p<0.05 vs control, Dunn's test for multiple comparisons. Mean±SEM. *(D)* Mander's correlation coefficient between DAPI and HSC70 signals for n=36 nuclei per condition. Kruskal-Wallis one-way ANOVA p<0.0001. *p<0.05 vs control, Dunn's Dunn's test for multiple comparisons. Mean±SEM. Black line between lanes indicates noncontiguous samples from the same blot.



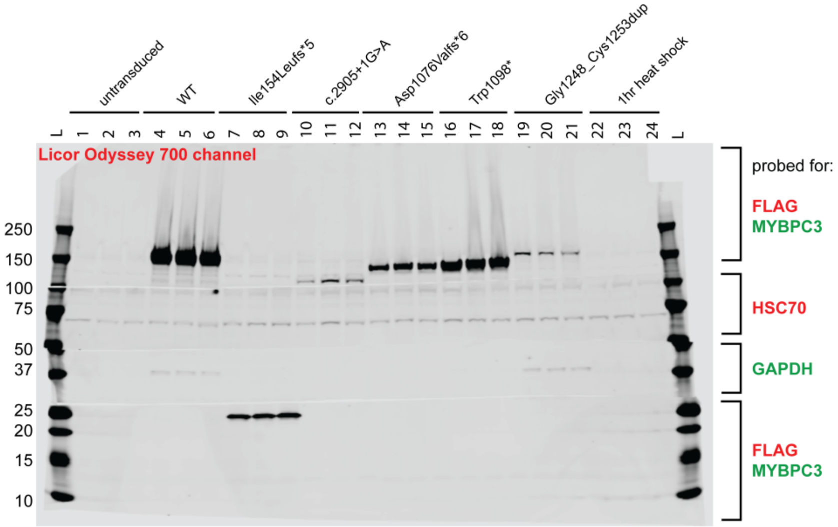
Supplemental Figure 7, cont. *(E)* Representative confocal images with Z-projections of patterned NRVMs immunostained for HSC70. Step size of 0.6μm. Scale bar = 20μm, 60X magnification.

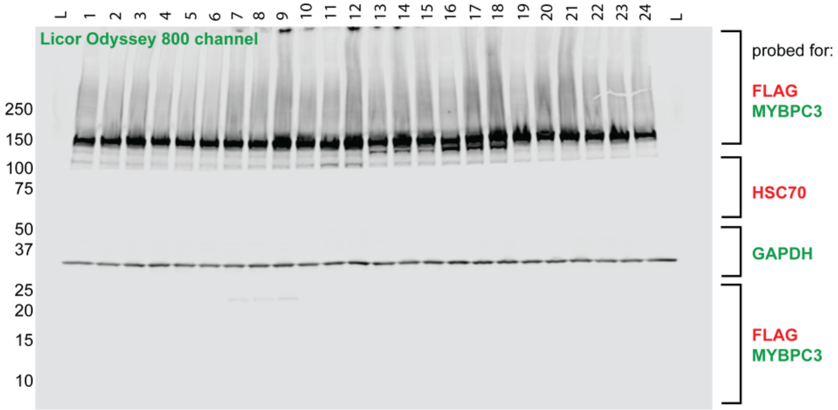


Supplemental Fig 8. HSP70 (*HSPA1A*) transcript *(A)* and protein *(B)* abundance in human myocardial tissue from donor controls and HCM patients (septal myectomy samples). *HSPA1A* mRNA abundance was normalized to two different internal reference genes (β2-microglobulin/*B2M* and 60S ribosomal protein L32/*RPL32*). No significant differences in HSP70 mRNA or protein were observed in tissue samples from HCM patients with *MYBPC3* mutations compared to controls or HCM patients with no identified sarcomere gene mutations. Mean±SEM, Kruskal Wallis one-way ANOVA p>0.05. *C)* Representative Western blot for HSP70. HSC70 (*HSPA8*) mRNA *(D)* and protein *(E)* abundance. No differences in HSC70 protein expression were observed. *HSPA8* mRNA was significantly reduced in both MYBPC3 and sarcomere-mutation negative HCM samples when normalized to *RPL32*, but not *B2M*. Mean±SEM, Kruskal Wallis one-way ANOVA; *p<0.0001, Dunn's test for multiple comparisons. *(F)* Representative Western blot for HSC70. Black line between lanes indicates noncontiguous samples from the same blot. For transcripts, healthy control n=11, MYBPC3 mutations n=11, non-sarcomere mutations n=12. For HSP70 protein, healthy control n=7, MYBPC3 mutations n=10, non-sarcomere mutations n=18. For HSC70 protein, healthy control n=7, MYBPC3 mutations n=6, non-sarcomere mutations n=7.

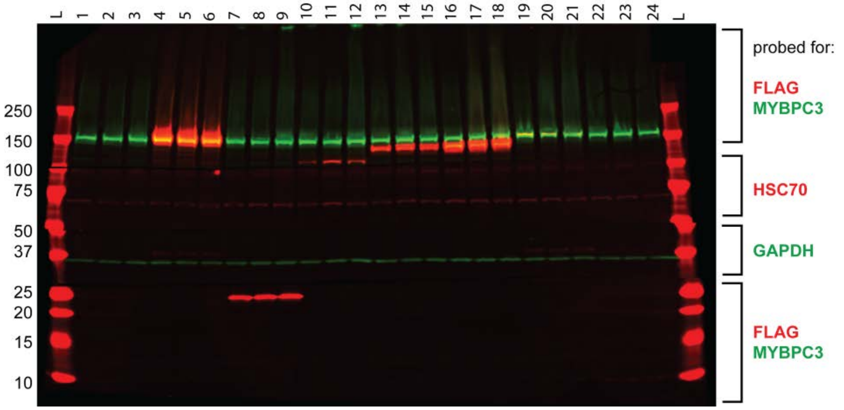
Full unedited blot for Figures 1B, S1E, and S7B

lanes used in Fig. 1B for FLAG and GAPDH: 4 - 21
lanes used in Fig. S1E for MYBPC3 and GAPDH: 1-21
lanes used in Fig. S7B for HSC70 and GAPDH: 2,3,4,5,8,9,11,12,13,14,17,18,19,20,22,23



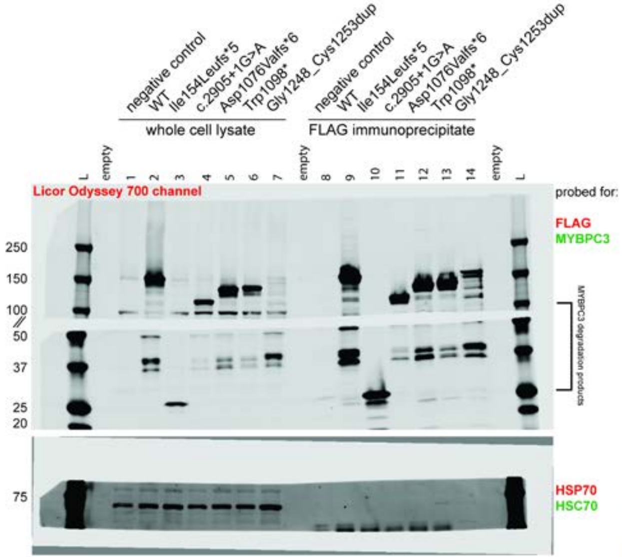


Combined channels image



Full unedited blot for Figure 2A

lanes used for FLAG, HSC70 and HSP70: 1-14



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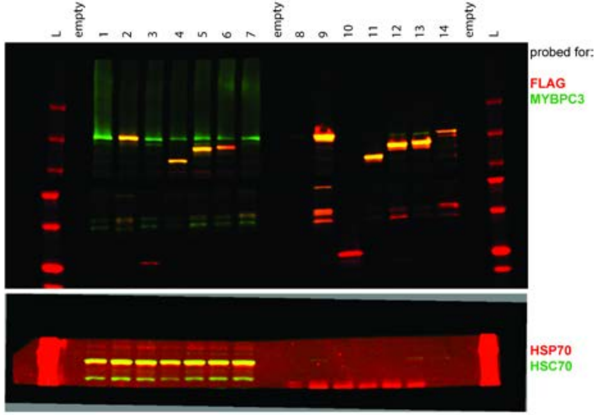
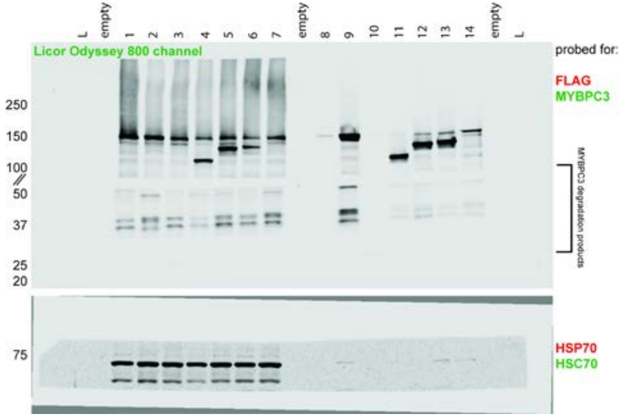
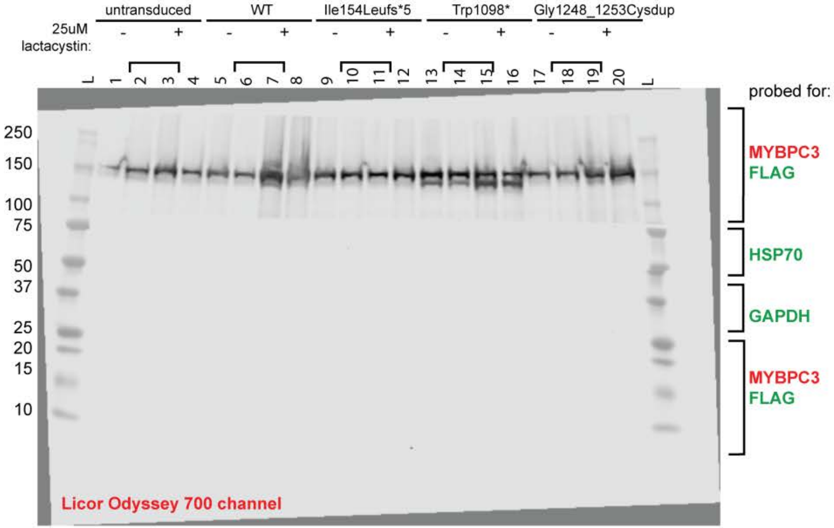




unedited blot for Figure 5C
lanes used: 2,3,6,7,10,11,14,15,18,19



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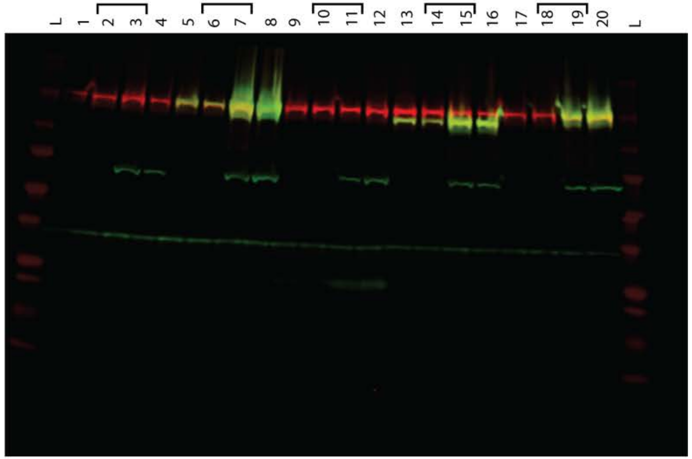
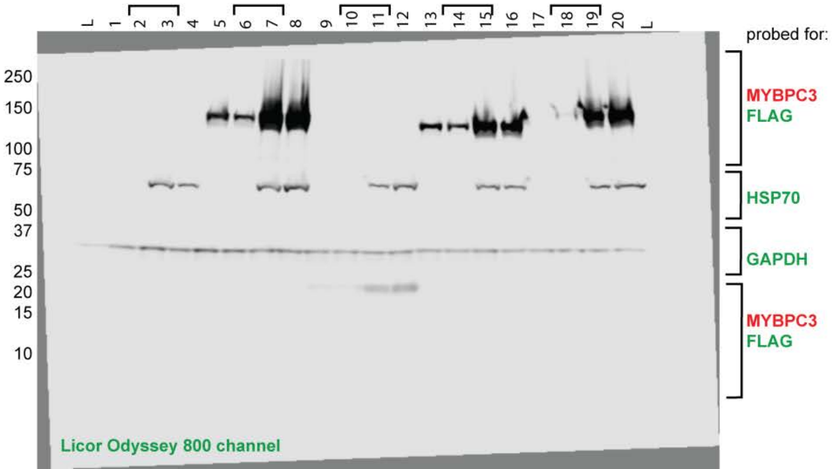
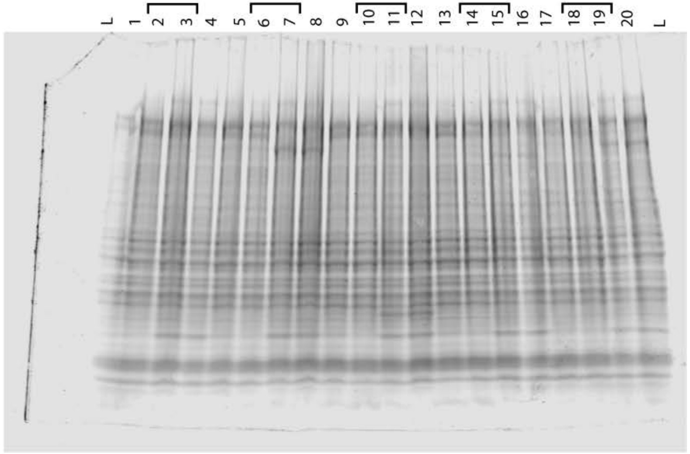


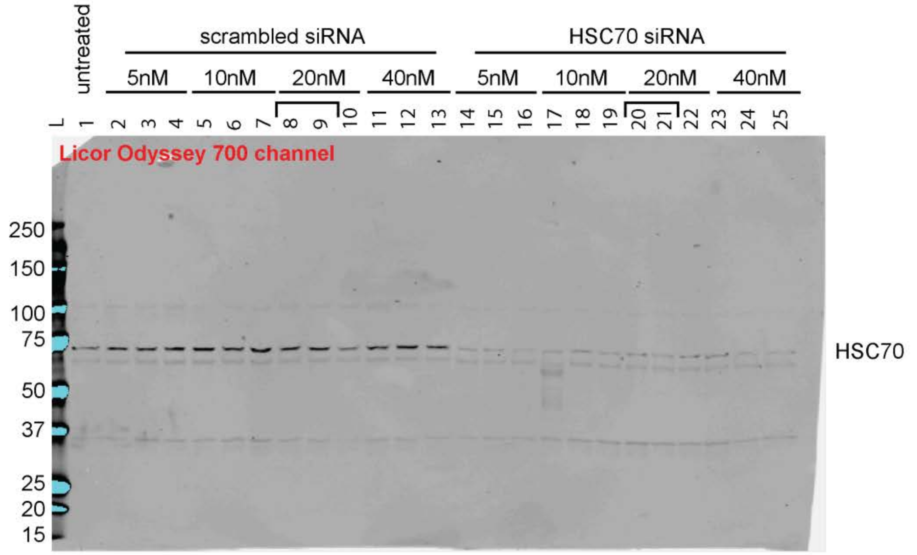


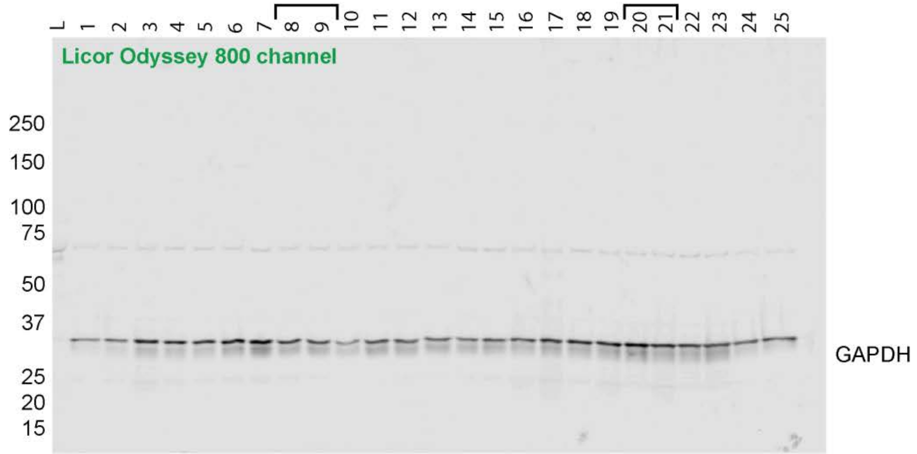
unedited Coomassie-stained gel for Figure 5C

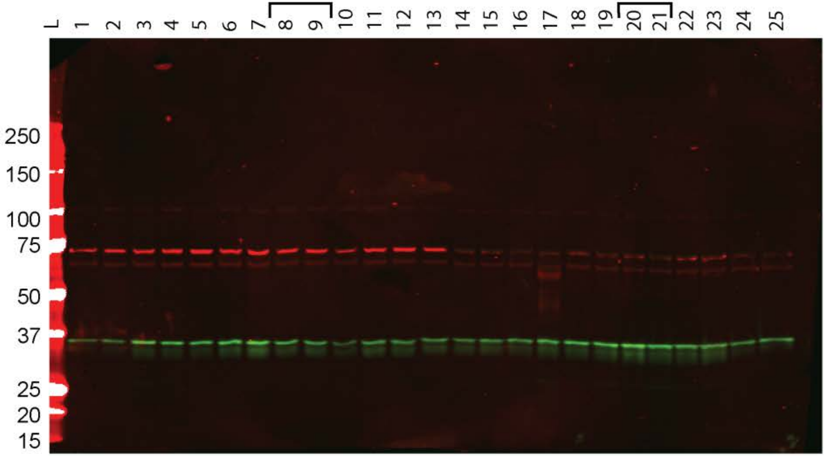


Full unedited blot for Supplemental Figure 2C

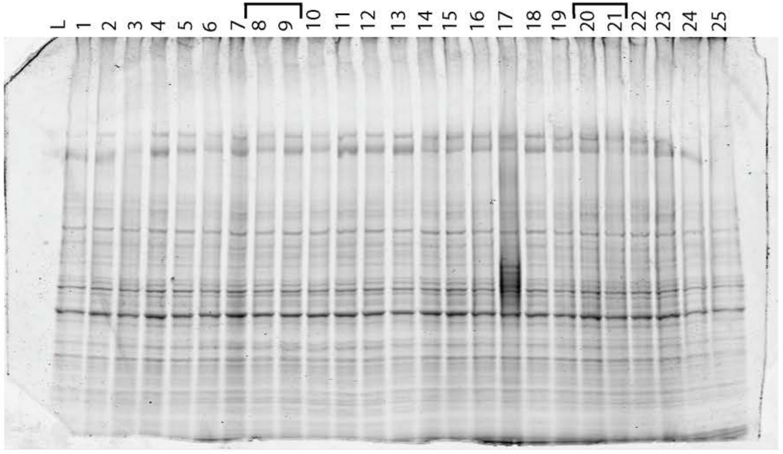
Lanes used for HSC70, GAPDH and gel: 8,9, 20, 21





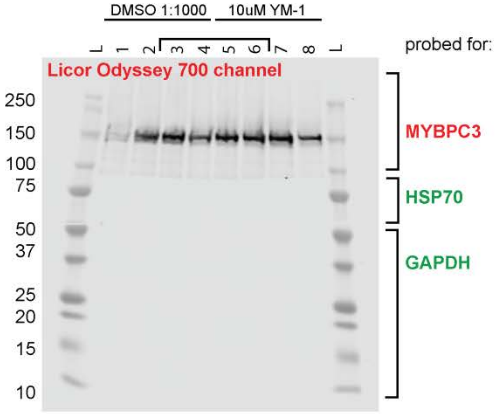


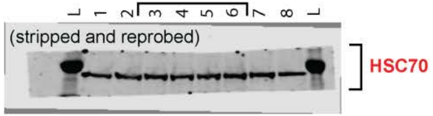
unedited Coomassie-stained gel

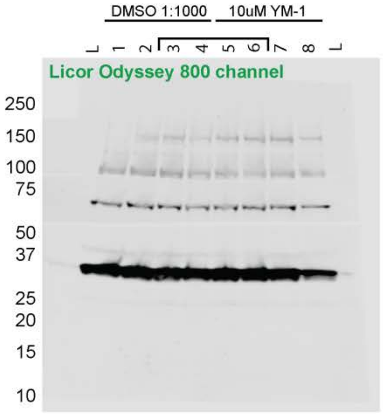


Full unedited blot for Supplemental Figure 2F

Lanes used for HSP70, HSC70 and GAPDH: 3 - 6







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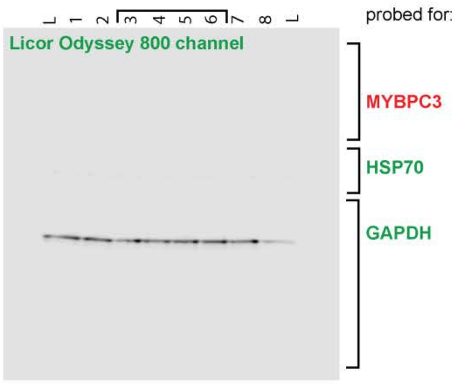
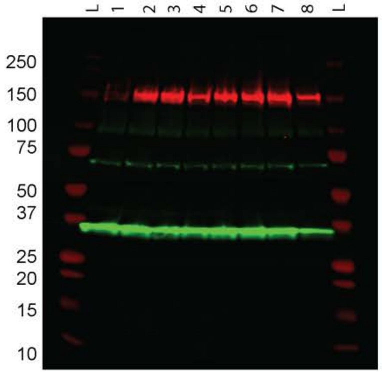
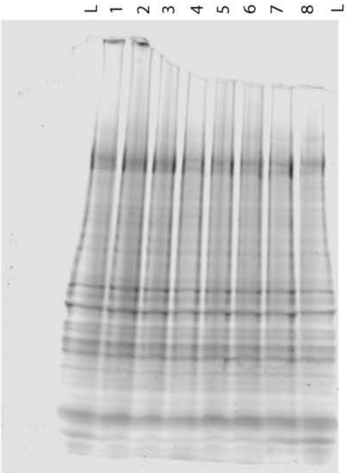


low exposure

Combined channels image

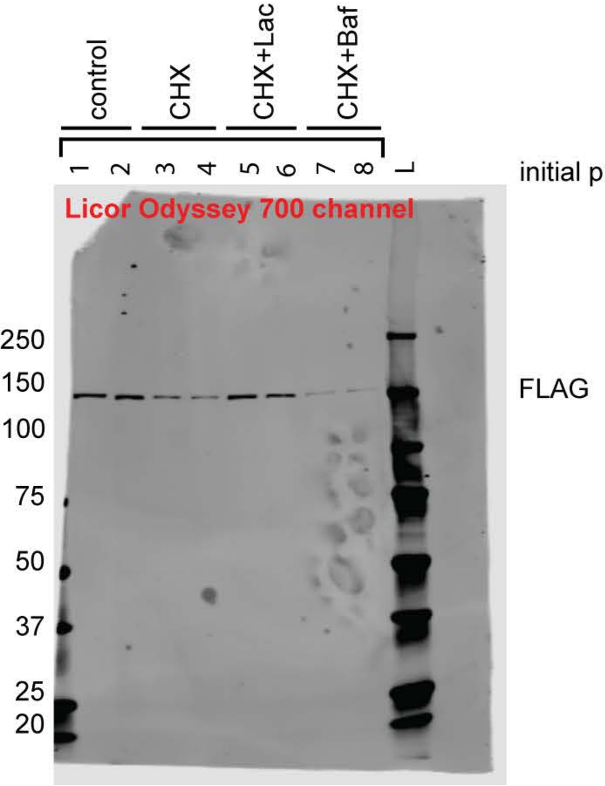


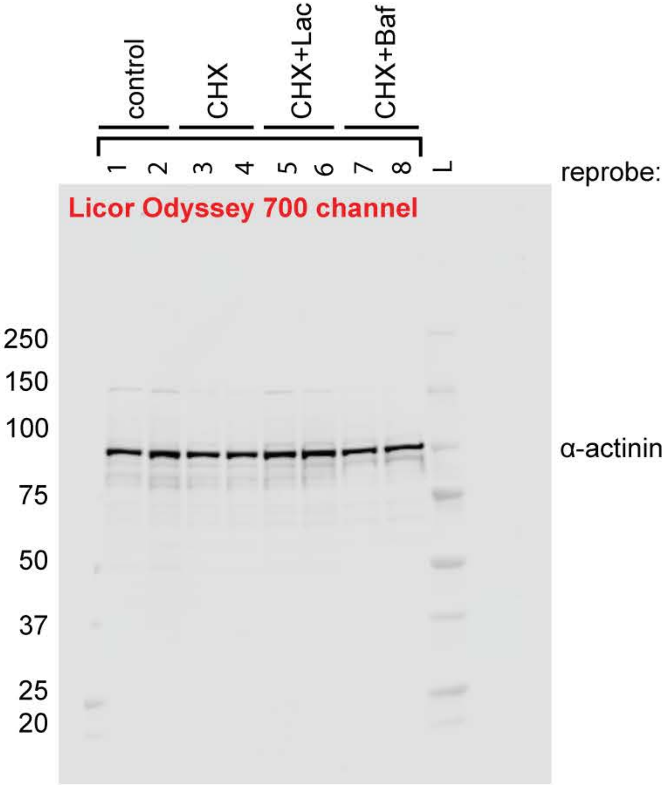
Full unedited Coomassie-stained gel

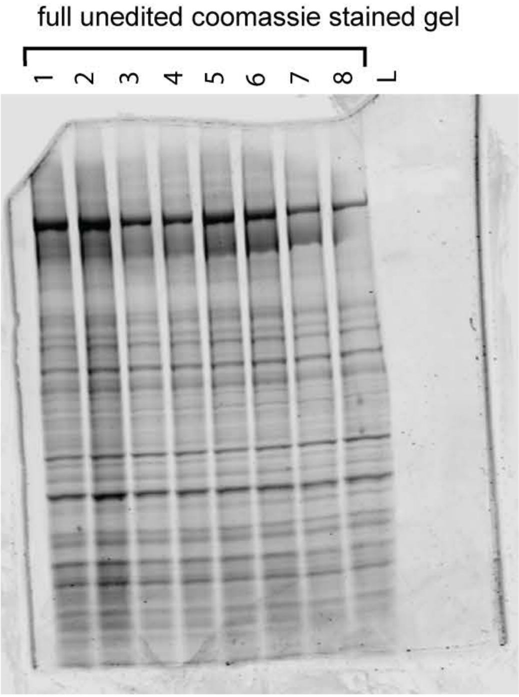


Full unedited blots for Supplemental Figure 2G

Lanes used: 1-8

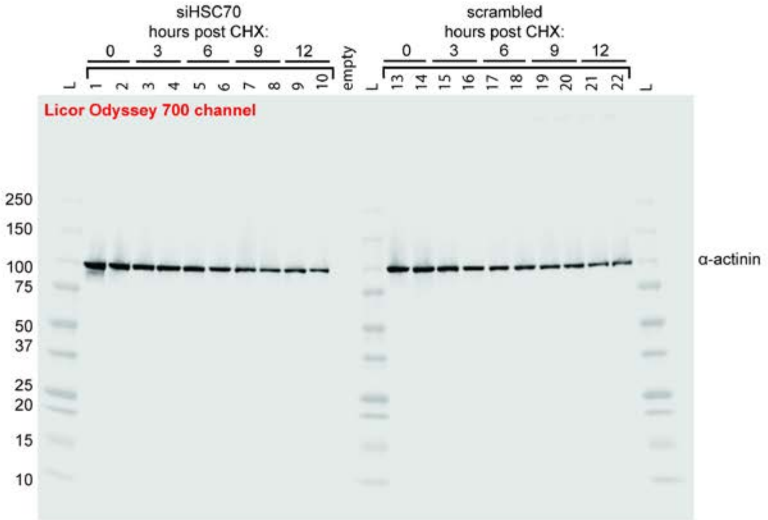




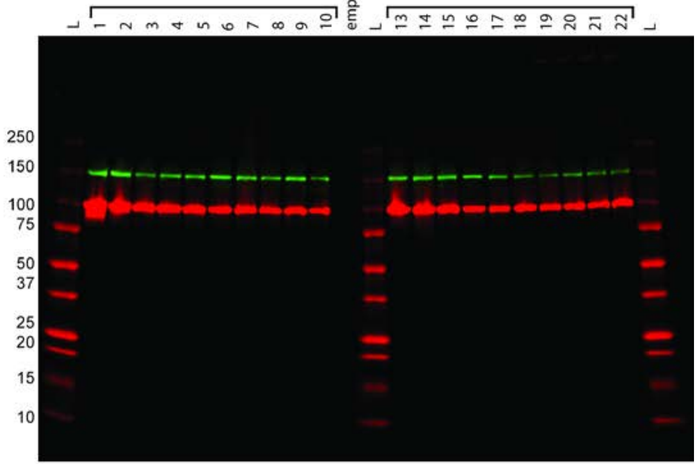


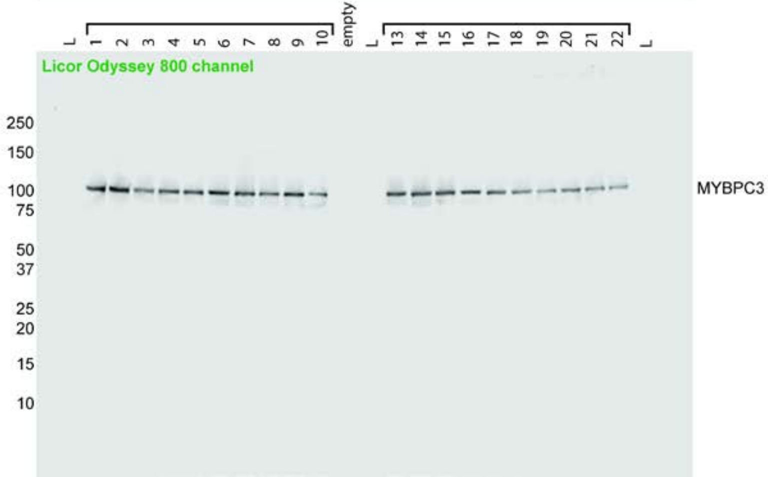
Full unedited blot for Figure S3A

Lanes used: 1-10, 13-22

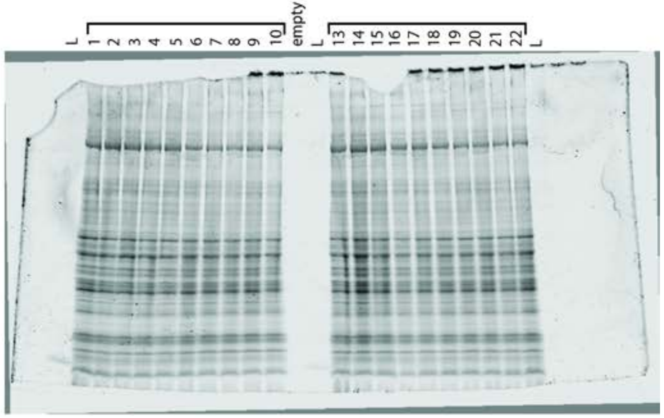


Combined channels image



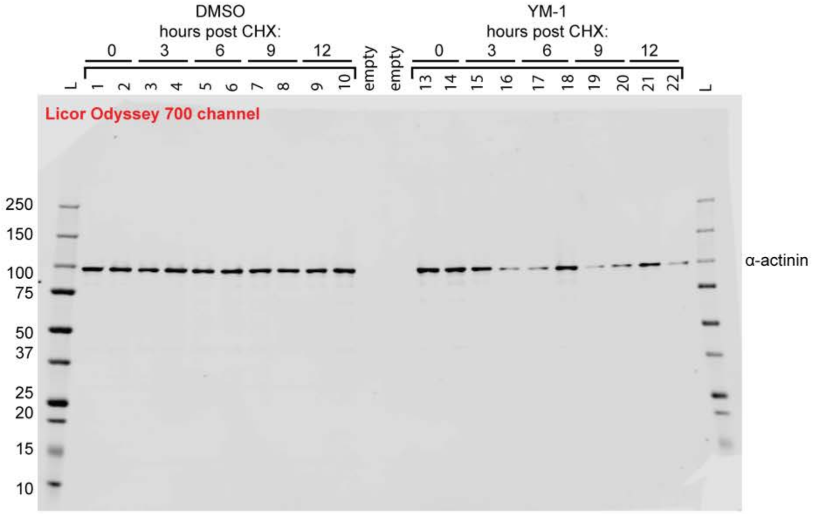


full unedited Coomassie-stained gel



Full unedited blot for Figure S3B

Lanes used: 1-10, 13-22



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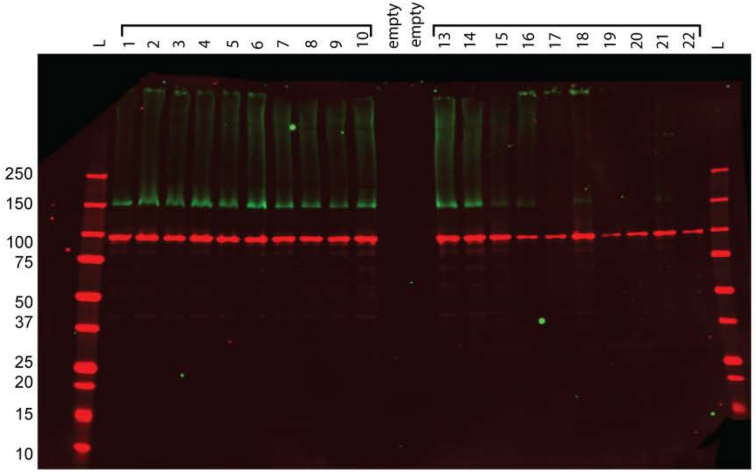
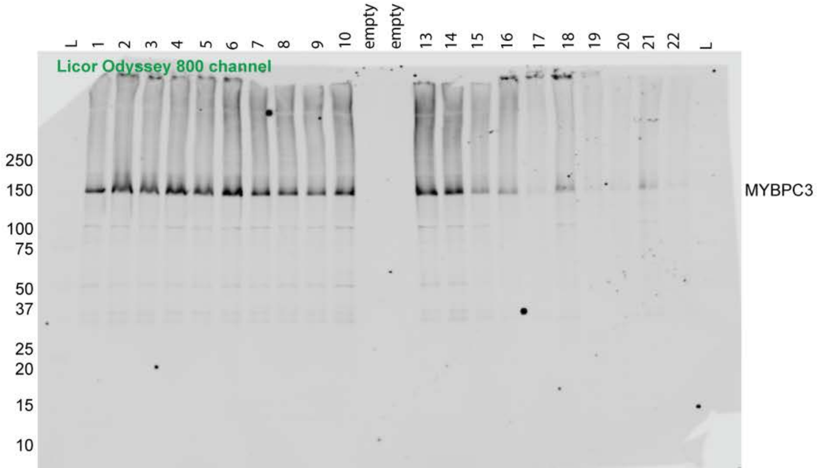
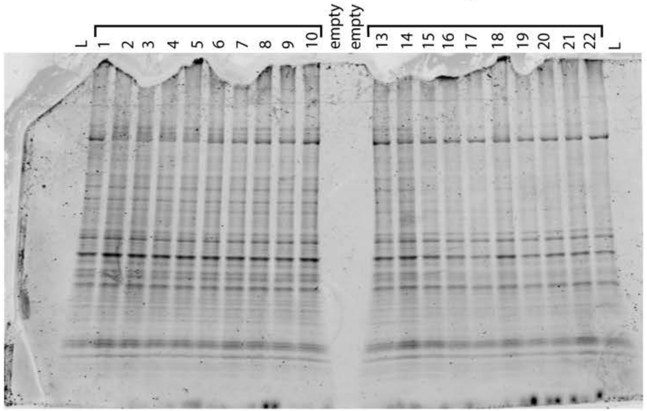


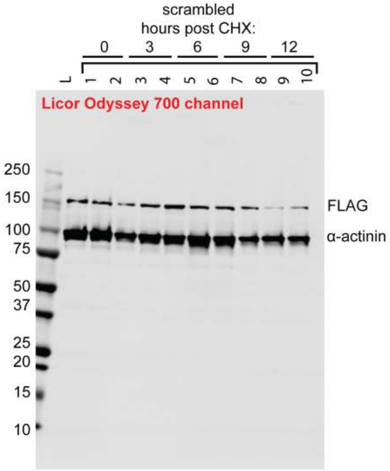


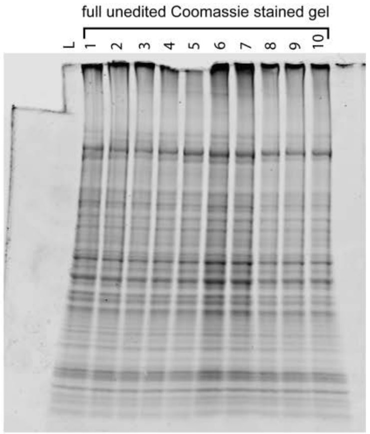
full unedited Coomassie-stained gel



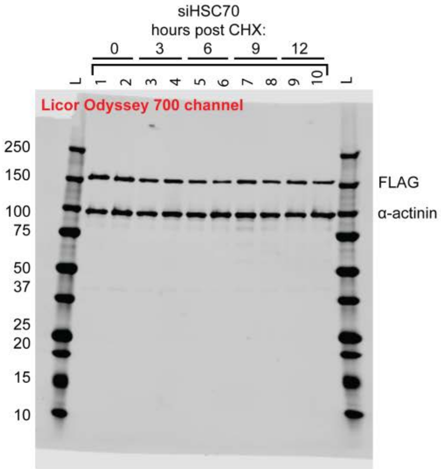
Full unedited blots for Figure S3C

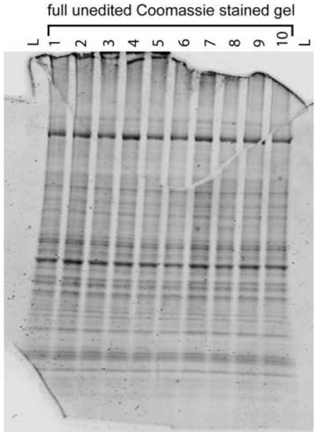
Lanes used: 1-10





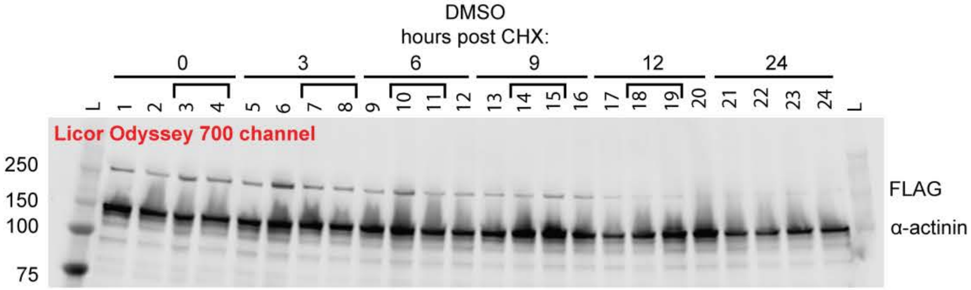
Lanes used: 1-10



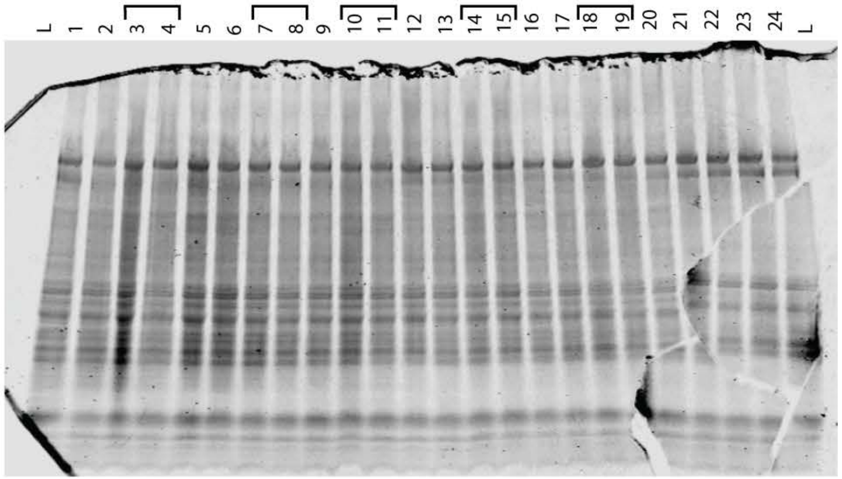


Full unedited blot for Figure S3D

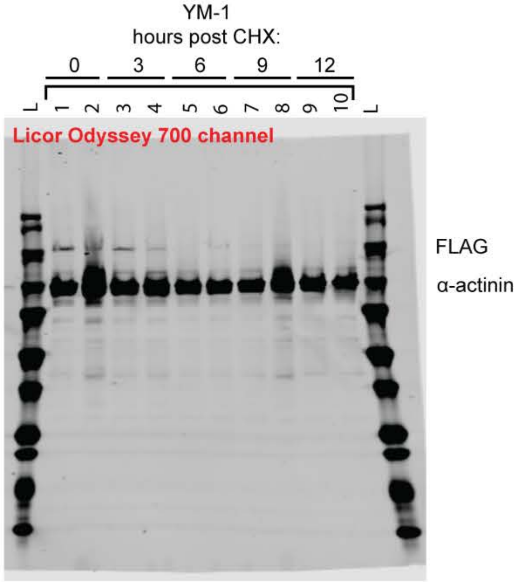
Lanes used: 3,4,7,8,10,11,14,15,18,19



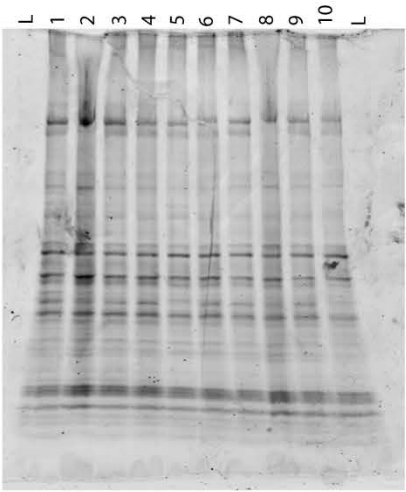
full unedited Coomassie stained gel



Lanes used: 1-10

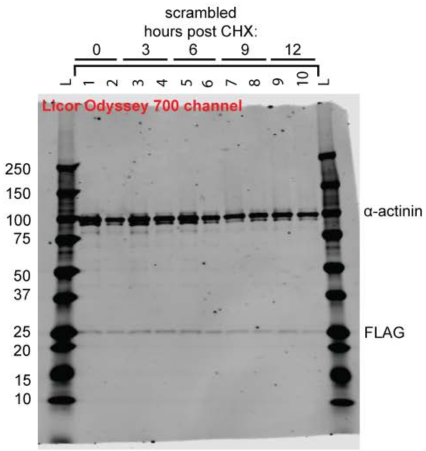


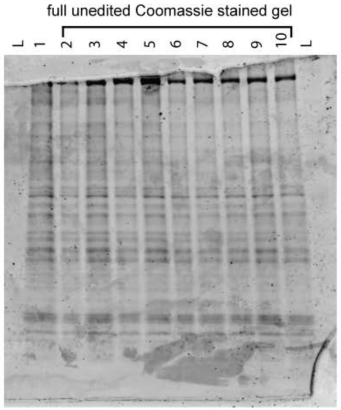
full unedited Coomassie stained gel



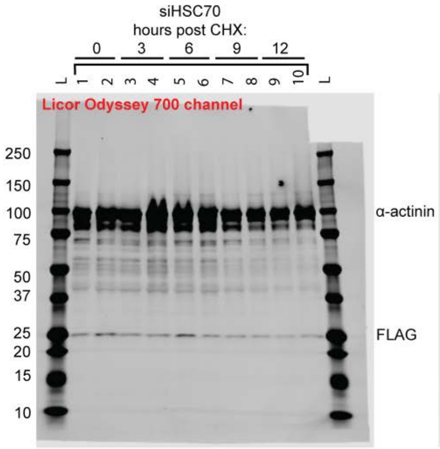
Full unedited blots for Figure S3E

Lanes used: 1-10

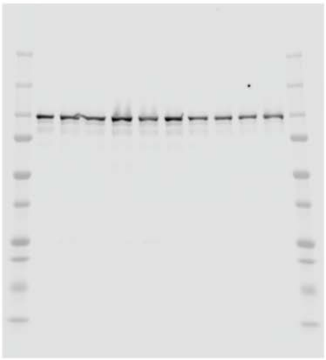




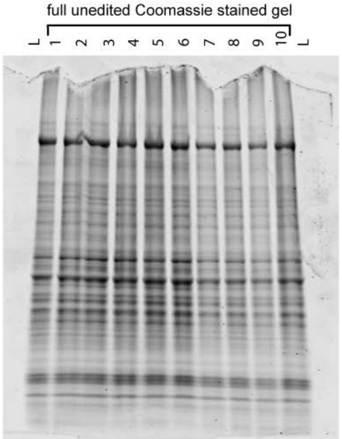
Lanes used: 1-10



high exposure

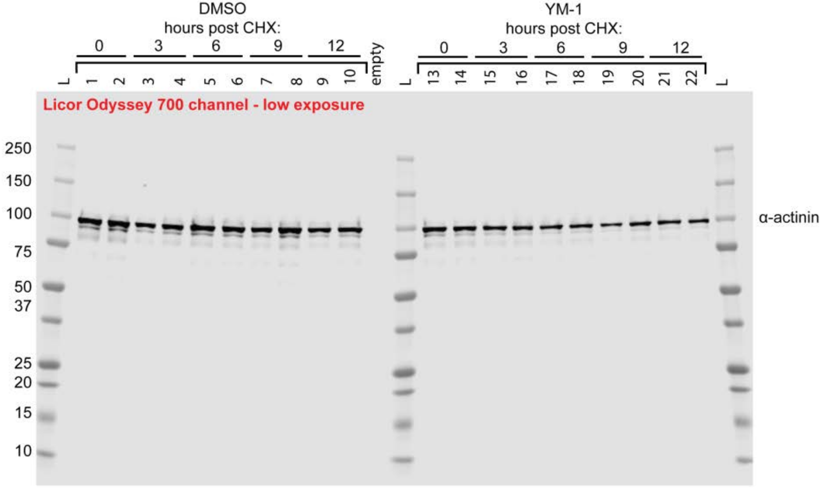


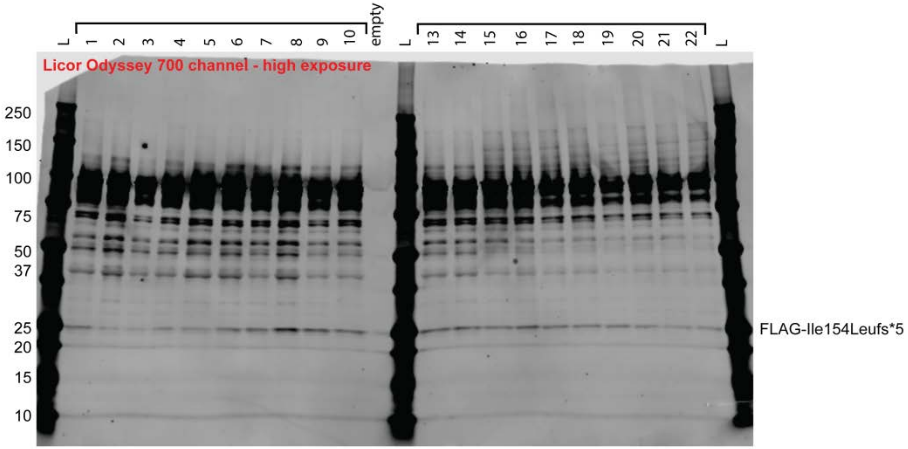
low exposure



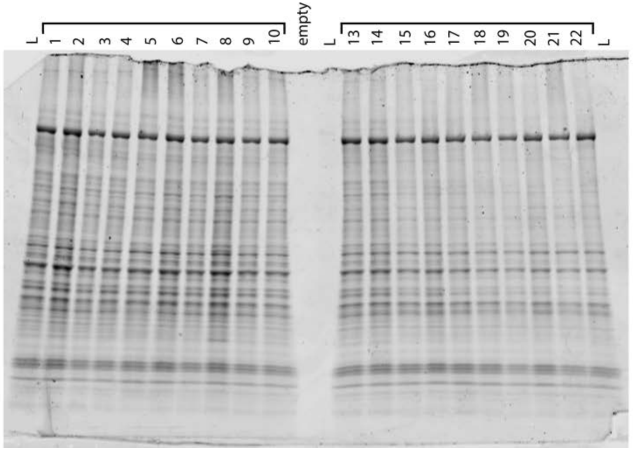
Full unedited blot for Figure S3F

Lanes used: 1-10, 13-22





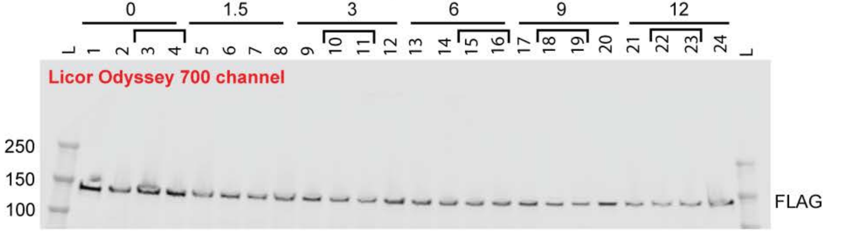
full unedited Coomassie stained gel



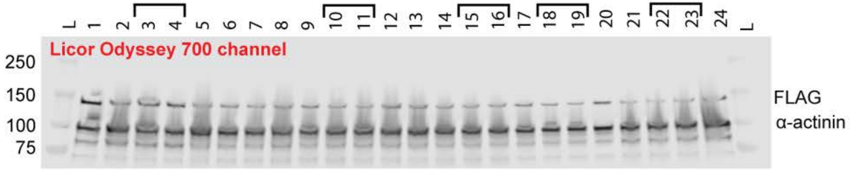
Full unedited blots for Figure S3G

Lanes used: 3,4,10,11,15,16,18,19,22,23

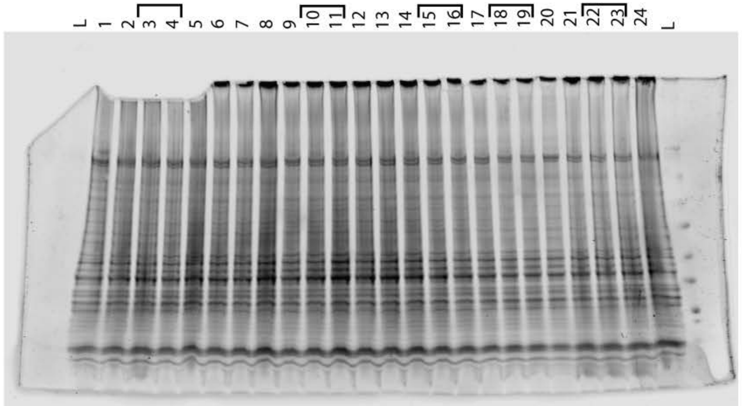
scrambled
hours post CHX:



α-actinin strip and reprobe

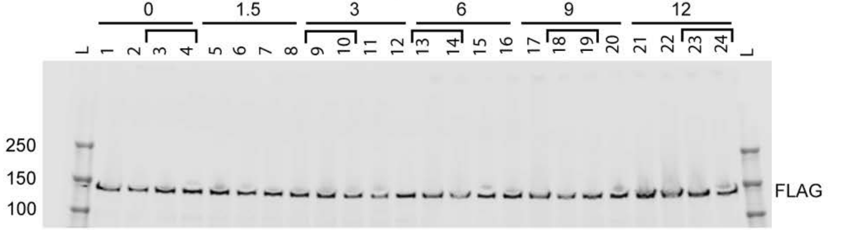


full unedited Coomassie-stained gel

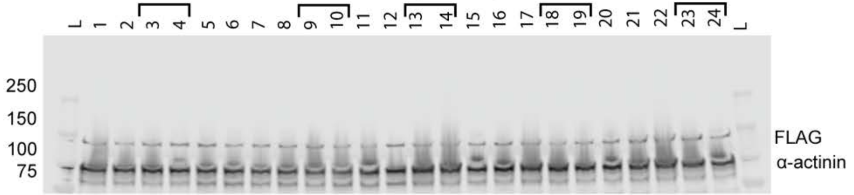


Lanes used: 3,4,9,10,13,14,18,19,23,24

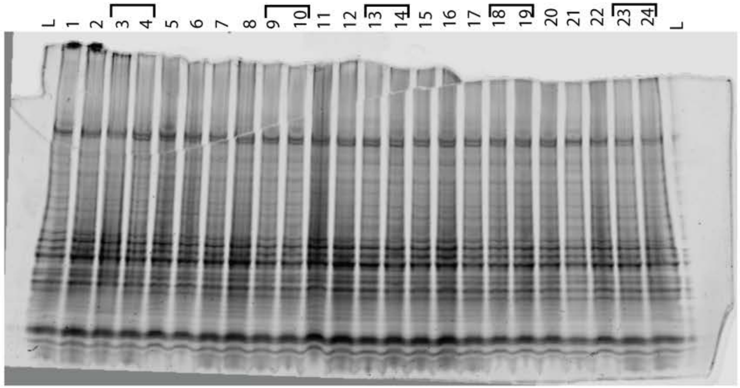
siHSC70
hours post CHX:



α-actinin strip and reprobe

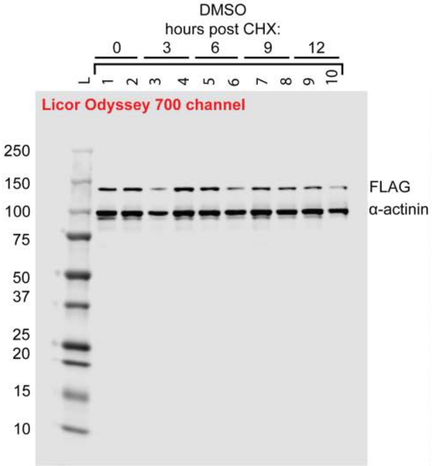


full unedited Coomassie-stained gel

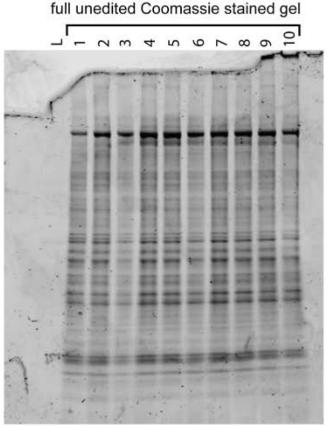


Full unedited blots for Figure S3H

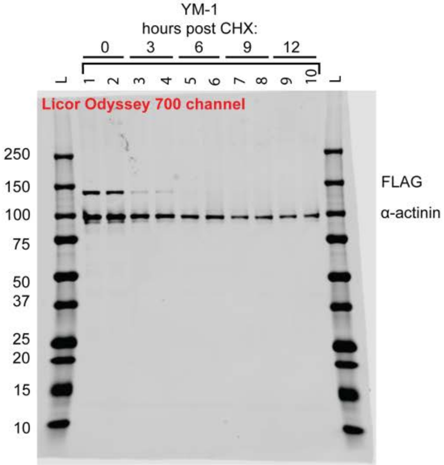
Lanes used: 1-10



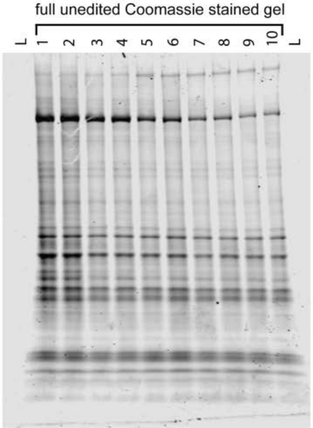
full unedited Coomassie stained gel



Lanes used: 1-10

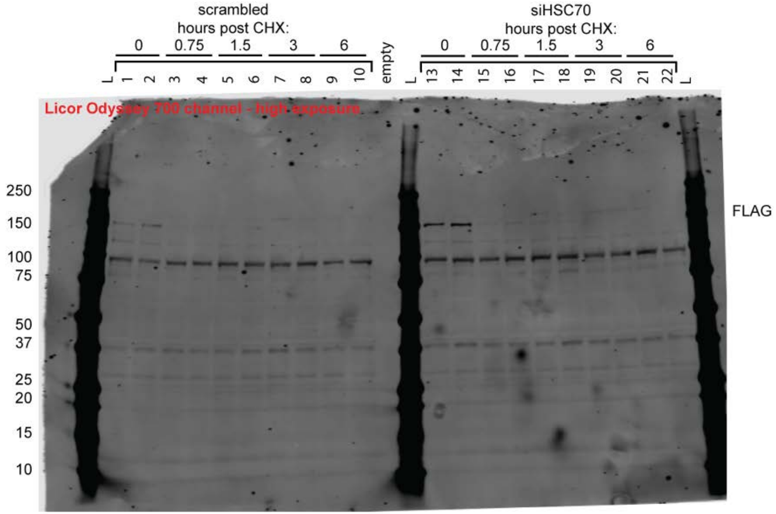


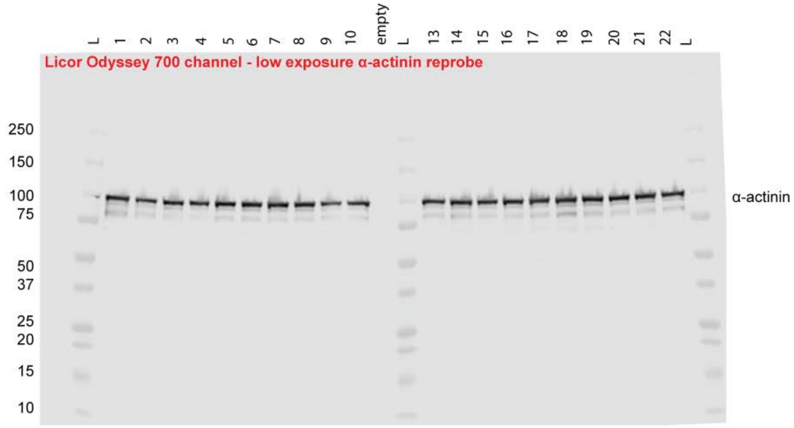
full unedited Coomassie stained gel



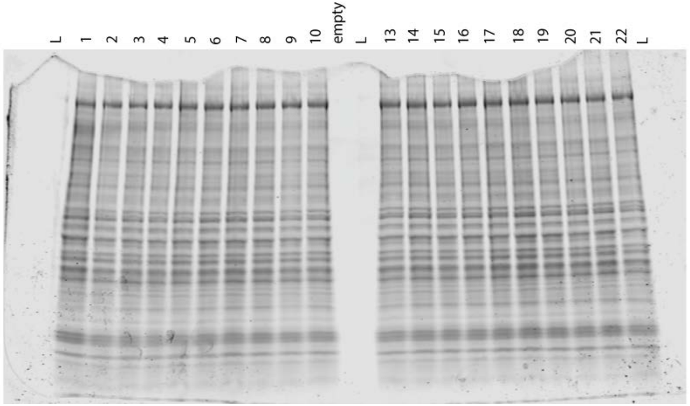
Full unedited blot for Figure S3I

Lanes used: 1-10, 13-22



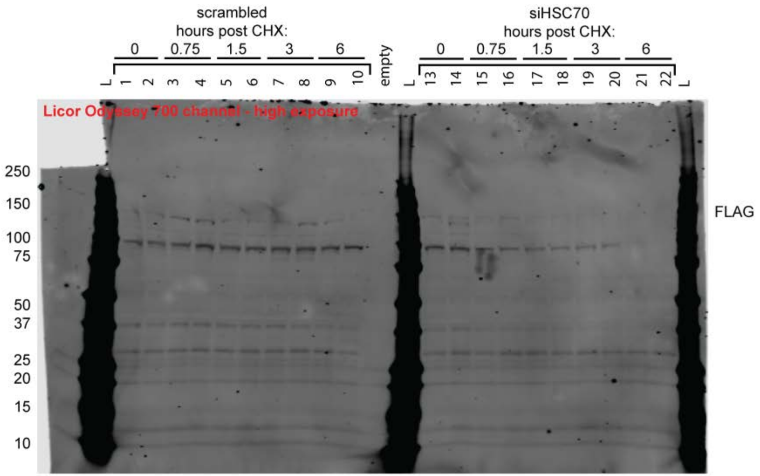


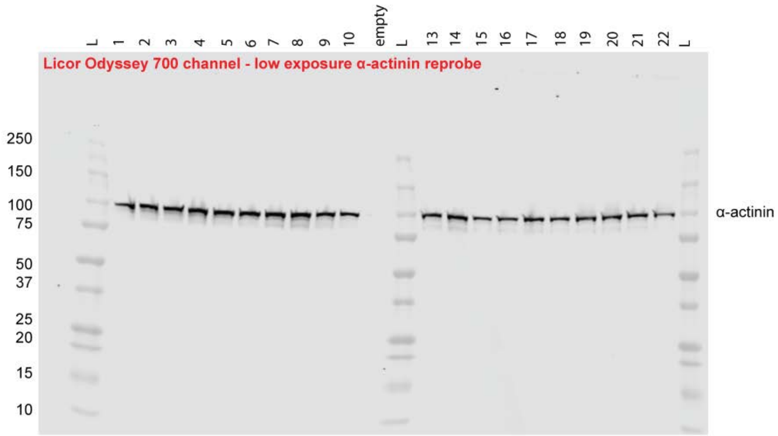
full unedited Coomassie stained gel



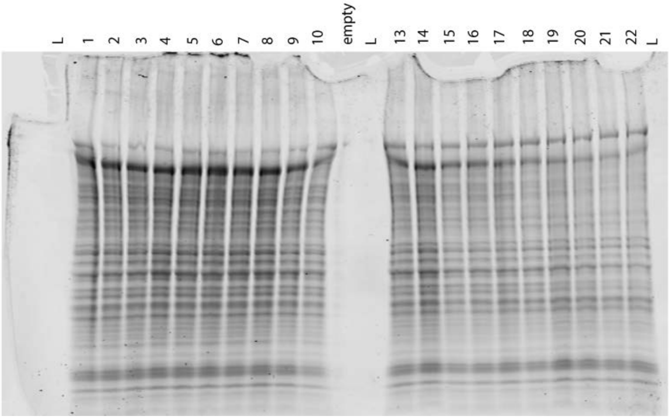
Full unedited blot for Figure S3J

Lanes used: 1-10, 13-22



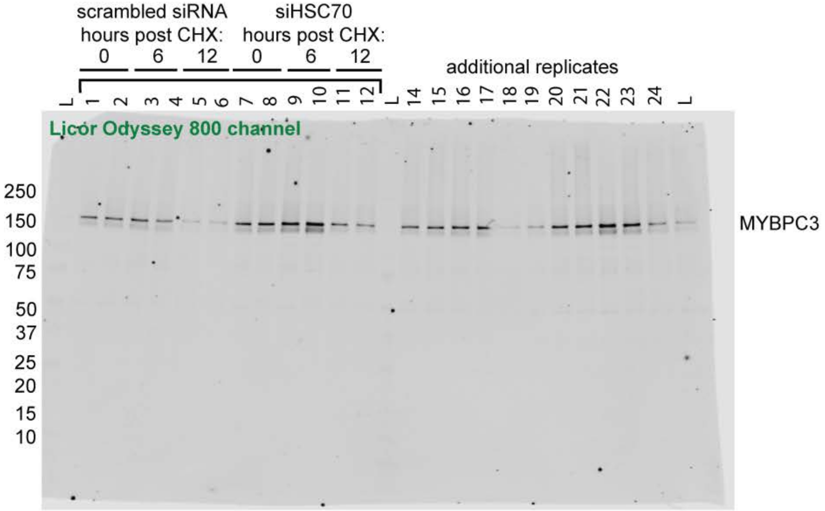


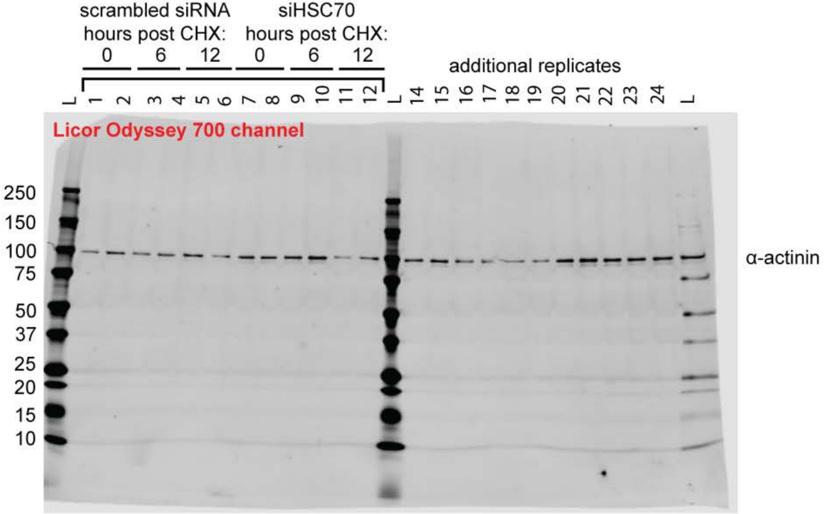
full unedited Coomassie stained gel

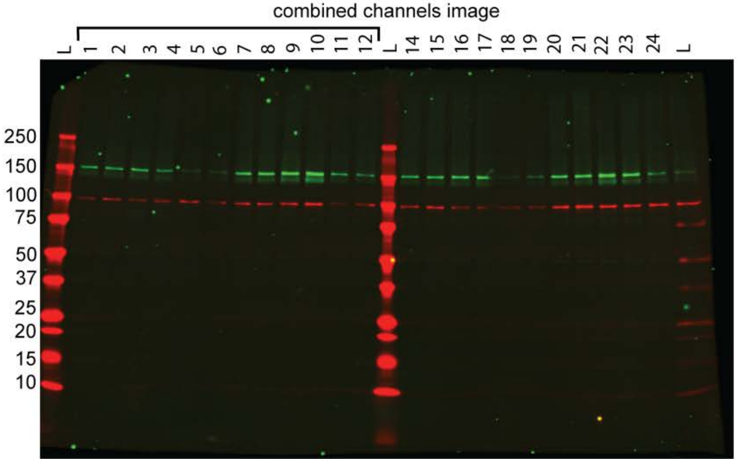


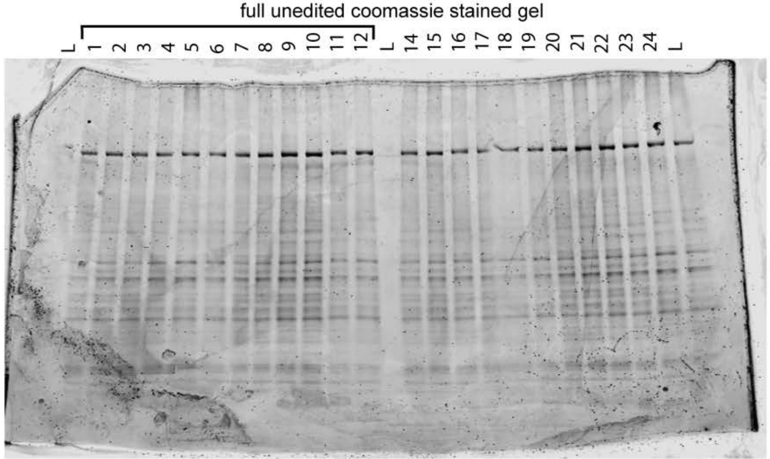
Full unedited blots for Supplemental Figure 5B

Lanes used: 1-12



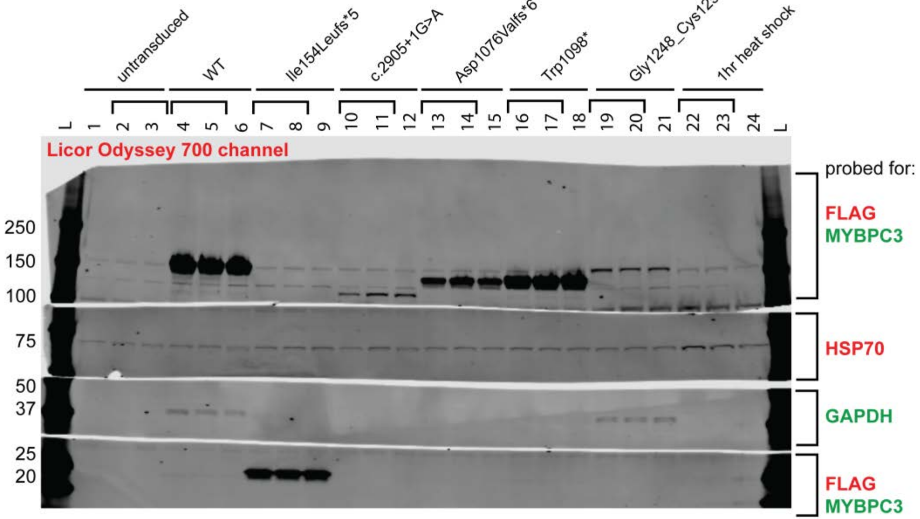


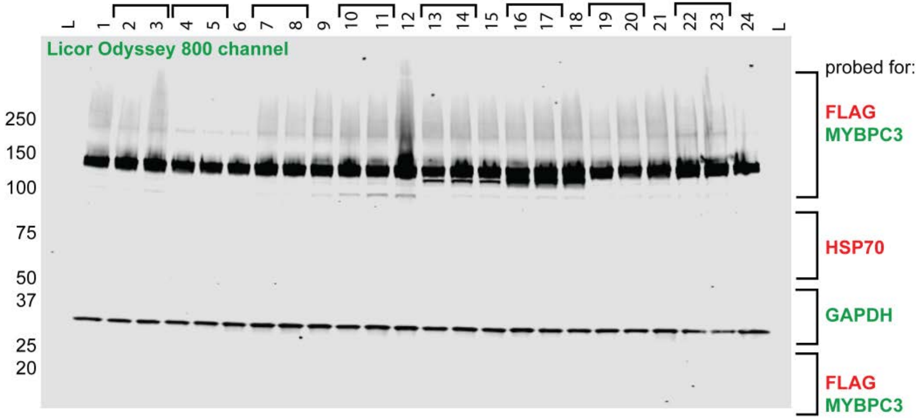




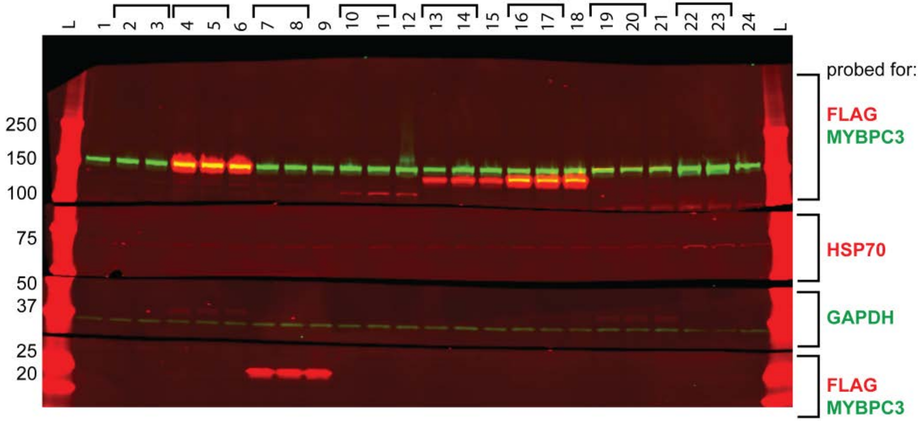
Full unedited blot for Supplementary Figure 7A

lanes used for HSP70 and GAPDH:
2,3,4,5,7,8,10,11,13,14,16,17,19,20,22,23



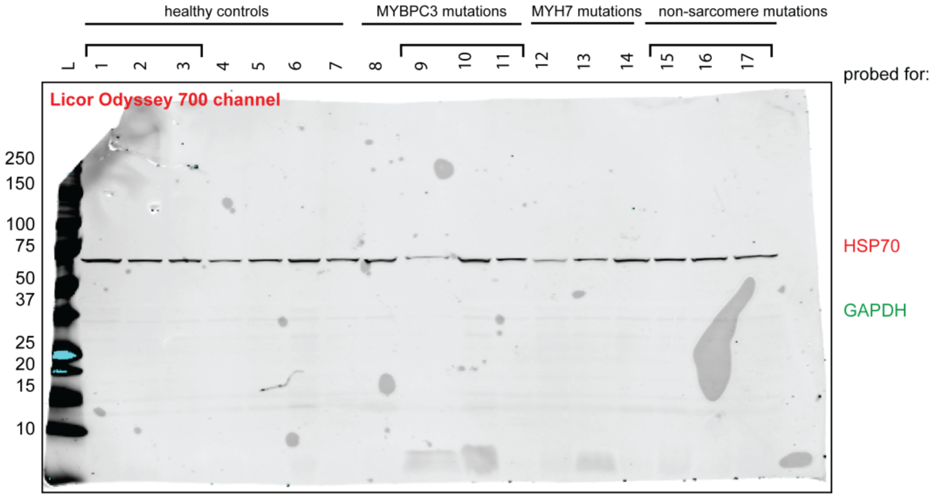


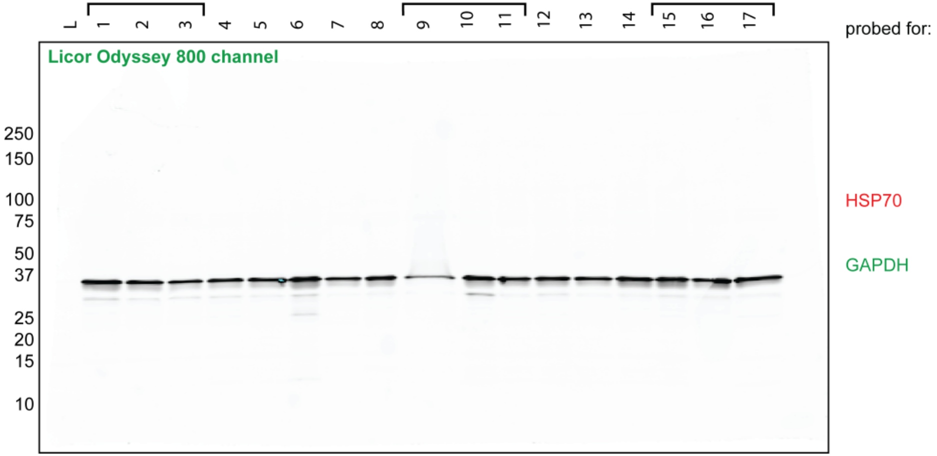
Combined channels image

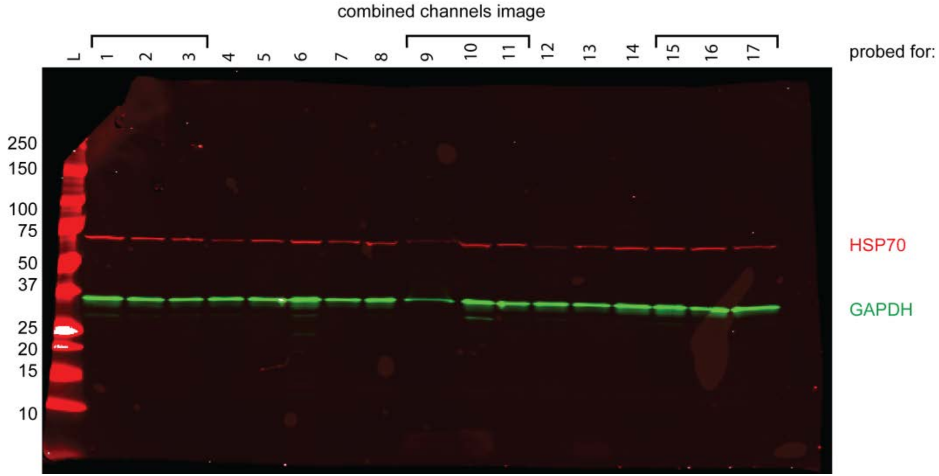


Full unedited blot for Figure S8C

Lanes used: 1-3, 9-11, 15-17







Full unedited blot for Figure S8F

Lanes used: 5-7, 10-12, 18-20