Supplementary Data

bHLH proteins involved in Drosophila neurogenesis display extensive mutual post-translational regulation

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Supplementary Figure Legends

Supplementary Figure 1: Sc[RQEQ] has lost its ability to bind onto DNA and activate transcription.
A: EMSAs using an E_{AB} oligonucleotide probe and in vitro translated proteins as indicated. 0: reticulocyte lysate mock reaction. Da homodimers form a high-MW complex (arrow), whereas Da/Sc heterodimers form a lower-MW complex (arrowhead). Asterisk: free probe. Note that Sc[RQEQ] has lost its ability to bind onto DNA, while Sc[m5p] binds onto DNA as efficiently as Sc.
B: Luciferase assays on transiently transfected S2 cells with an EE4-luc reporter. Activation levels by cotransfected Da and increasing amounts of myc-Sc or myc-Sc[RQEQ]. Relative luciferase units (rlu) are shown and are normalized on the activity of the reporter gene alone, set to 1. Averages/standard deviations of triplicates are shown.

Supplementary Figure 2: Sc is subject to multiple phosphorylations which are variably affected by kinase inhibitors LiCl and Senexin A.
A. Whole cell extracts from S2 cells transfected with the indicated expression plasmids and treated with LiCl for 4 hours before lysis. Note that Da expression produces a doublet of high MW species (β, β'), as well as a hyperphosphorylated form (α') running slightly slower than (and barely resolved from) the major band (α). Low levels of the unphosphorylated form (u) are seen in the absence of Da. Li treatment slightly increases the u form in the absence of Da and reduces the α' and β' forms in the presence of Da. The α and β major bands are not strongly affected. The major band of Sc[m3p] is the unphosphorylated form. Upon Da coexpression a phosphorylated u' band is seen, which is diminished by Li treatment. **B.** Cotransfection of gradually increasing amounts of Da enhances the β, β' and α' forms of Sc. Sc[S217A] does not show the α' and β' bands, whereas Sc[T329V] does. The double phosphomutant Sc[m2p] also lacks the α' and β' bands; none of the phosphomutants affects the β modification. In these blots the amount loaded was adjusted according to the activity of luciferase expressed from control plasmid Ract-luc cotransfected with the indicated plasmids (to measure transfection efficiency). **C:** λ-phosphatase treatment of S2 cell extracts expressing myc-Sc protein variants along with Da. i: input, λ: treated, m: mock-treated. Note the decrease of α', β' and u' bands, but the persistence of the β band and the increase in the u band. Together with the results of panels A and B, this suggests that the α', β' and u' bands probably represent Sgg-phosphorylated species, which depend on S217, whereas the β form is not due to phosphorylation. **D:** Whole cell extracts from S2 cells transfected with myc-Sc or myc-Sc[1-320], with or without cotransfected His-Da and treated differentially with the Cdk8 inhibitor Senexin A and the proteasomal inhibitor (MG132) as depicted in the experimental scheme. Note that treatment of transfected S2 cells with Senexin A caused a severe reduction of the β/β' forms of Sc and Sc[1-320]. **E:** Equal amounts of S2 cells treated as panel D were blotted for Arm levels. Note that Senexin A causes a reduction in Arm levels, whereas proteasome inhibition increases Arm levels.

Supplementary Figure 3: Estimation of the half-life of Sc and its variants. A, C: Western blots of S2 cell extracts. Cells had been treated with cycloheximide and lanes are labelled as in Fig. 1D. Myc-tagged protein lavels were estimated by densitometry and normalized against Gro. **B, D** show the estimated protein levels at each time-point and the half-lives of each variant. Each measurement was repeated several times; one indicative experiment is shown. The half-lives estimated (number of repeats in parenthesis) are: Sc 40±10 min (n=4); Sc[1-320] 127±12 min (n=5), Sc[m3p] 75±4min (n=4), Sc[RQEQ] 60±20 min (n=8), Sc[1-318]-LH 227±104 min (n=2), Sc[1-318]-AD1 78±11 min (n=2).

Supplementary Figure 4: Da does not affect the turnover of Sc variants that lack the bHLH domain or the SPTSS motif.
In all panels N-terminally 3xmyc-tagged Sc proteins or His-tagged Da were detected. The amount loaded was adjusted according to the activity of luciferase expressed from control plasmids (Ract-luc) transfected at the same time as the indicated plasmids (to measure transfection efficiency).
A: Steady state levels of myc-GFP with increasing amounts of Da. GFP is not affected by increasing levels of Da.
B : Steady state levels of Sc[164-345] and Sc[1-163] with increasing amounts of Da. Neither is affected by increasing Da.
C: Steady state levels of Sc[1-320] or Sc[1-320, m3p] with increasing amounts of Da with or without treatment of MG132 for ~8-10 hours before lysis. Note that Sc[1-320] levels drop by increasing Da and proteasome inhibition abolishes this decrease, while Sc[1-320, m3p] is not affected by Da.

Supplementary Figure 5: The SPTSS motif and Sc-E(spl) interactions are necessary for an E(spl) protein to promote Sc turnover.
A,B, D-H: Whole cell extracts from S2 cells transiently co-transfected with the indicated plasmids. The amount loaded was adjusted according to the activity of co-transfected actin driven luciferase (or hs driven β-galactosidase, for blot 5D). **A:** S2 cells transfected with the myc-tagged version of m7, m7KNEQ, m7ΔW or m7EQRQTKHQ (quadruple point mutation in the Orange domain, marked OR*). The expression levels of all m7 variants are similar. **B**: S2 cells co-transfected with myc-Sc[m5p] and increasing amounts of myc-m7. The upper band in the anti-myc blot is Sc[m5p] (arrowhead), whereas the lower one is m7 (arrow). **C:** myc-tagged E(spl) proteins (as indicated; "wt" is wt m7, OR* is m7EQRQTKHQ) were immunoprecipitated from S2 cells. Left panels: Immunoprecipitation efficiency (anti-Myc blot) and the presence of coimmunoprecipitated endogenous Gro (a-Gro) were assayed. Input levels of endogenous Gro is shown in the upper panel. m7ΔW and GFP do not interact with Gro, while m7, m7KNEQ and m7EQRQTKHQ interact with Gro. Note that m8 can extremely efficiently coimmunoprecipitate endogenous Gro. Right panels: Immunoprecipitation from cells coexpressing the indicated myc-tagged proteins and His-Da. Immunoprecipitation efficiency (a-myc panel) and the presence of coprecipitated His-tagged Da (a-His panel) and endogenous Gro (a-Gro) were assayed. m7ΔW interacts with His-Da, but not with Gro, while the Orange mutant m7EQRQTKHQ interacts with Gro, but not with His-Da. **D:** S2 cells co-transfected with myc-Sc and increasing amounts of E(spl)mγ, E(spl)mδ or E(spl)m7. **E:** S2 cells co-transfected with myc-Sc[1-320] and increasing amounts of myc-m7 Sc[1-320] was not susceptible to degradation by m7. **F**: S2 cells co-transfected with myc-Sc[1-

318]-AD1 and increasing amounts of myc-m7,myc-m7ΔW or myc-m7KNEQ. Sc[1-318]-AD1 was degraded by m7 and m7KNEQ, but not by m7ΔW. **G:** Sc[1-318]-LH was not destabilized by m7. **H:** Upon Da coexpression, both Sc and Da are destabilized by m7 and the β form of Sc becomes prominent. Note also a retarded form of m7 (upper arrow). In the blots of panels B and G, cells were cotransfected with Ract-lacZ and blotted for anti-β-gal. Note that lacZ levels are unaffected by m7 expression, showing the specificity of Sc degradation by m7. Asterisks in E and G: half amount of the adjacent sample is loaded for quantity validation.

Supplementary Figure 6: E(spl)m7 is ubiquitylated, SUMOylated, phosphorylated and is degraded via a proteasome-independent mechanism.
A: S2 cells were transfected with the indicated His tagged m7 variants. "His" (last lane) is a His-tagged short peptide used as control. Cells were lysed under strong denaturing conditions and His-tagged proteins were Ni-NTA affinity purified and blotted with ab-P4D1 (anti-Ubi), anti-SUMO or anti-RGS-His to detect input protein. His-m7[m2K_mut] has a cloning induced frameshift mutation at the 3' end, which generates a longer protein. m7 displays di (asterisk) and tri (arrow)-SUMO modifications, as well as mono- (asterisk), di- (arrow) and poly Ubi adducts. These modifications do not depend on Gro interaction or on K51, K54 or K72, as they are present in the His-m7ΔW and the His-m7[m2K_mut], [K72R] and [m3K] variants. **B-E:** Whole cell extracts from S2 cells transiently co-transfected with the indicated plasmids. The amount loaded was adjusted according to the activity of co-transfected actin driven luciferase. **B:** (upper panel) myc-m7 and two mutants, myc-m7EQRQTKHQ (OR*) and myc-m7ΔW were detected without or with proteasome inhibition my MG132. No significant stabilization is seen for any of the m7 variants upon proteasome inhibition. (Lower panel) Drosophila S2 cell extracts transiently co-transfected with myc-m7 and increasing amounts of myc-Sc, in the presence of DMSO or MG132 for 7 hours. Although in the absence of Sc MG132 has no effect on m7 levels, in the presence of Sc it clearly reduces m7 levels. This result could be explained by the stabilization effect that MG132 has on the Sc protein (see also Fig. 3) and a non-proteasomal route of m7 degradation, enhanced by increased Sc. **C:** λ-phosphatase treatment of S2 cell extracts coexpressing myc-Sc and myc-m7. Treatment increased the mobility of both myc-Sc and myc-m7. **D,E:** S2 cells were co-transfected with myc-m7EQRQTKHQ (OR*, arrow) and increasing amounts of myc-Sc or Sc[1-320] (arrowhead) in the absence or presence of His-Da. unt: untransfected control.

Supplementary Figure 7: Sc levels and activity are post-transcriptionally modulated in vivo.
A,B: All panels show parts of wing disks carrying one copy of the EE4-lacZ reporter and overexpressing myc-Sc proteins under the act5C promoter in clones, marked by GFP coexpression. Immunostaining: Sc (anti-myc, blue), β-galactosidase (anti-β-gal, red) and GFP (anti-GFP, green). **B:** Detail of the region boxed in **A**. Note that Sc levels (blue) and transcriptional activity (EE4-lacZ activation, red) are not concordant. Also note variable expression of Sc compared to the more uniform GFP.
C,D: All panels show parts of wing disks overexpressing myc-Sc proteins (anti-myc, red) in clones, marked by GFP coexpression (same genotype as A,B) and counterstained for endogenous E(spl) proteins (blue); the anti-E(spl) antibody recognizes five out of the seven E(spl) proteins (90). **D:** Detail of the region highlighted in **C**. Note that on a nucleus-by-nucleus basis, the levels of Sc

accumulation do not correlate with the levels of E(spl) accumulation, instead in many cases they are inversely correlated.

Supplementary Materials and Methods

3xmyc tagged constructs in Ract vector		
	Amino acid changes	Reference
myc-DaΔAD1	Deletion of aa 1-264	Zarifi 2012 and this work
myc-DaΔLH	Deletion of aa 211-463	Zarifi 2012 and this work
myc-DaΔTADs	Deletion of aa 1-414	Zarifi 2012 and this work
myc-Da	Wild type	Zarifi 2012 and this work
myc-Sc	Wild type	Kux 2013 and this work
myc-Sc[1-320]	Deletion of aa 321-345	Giagtzoglou 2005 and this work
myc-Sc[1-260]	Deletion of aa 261-345	Giagtzoglou 2005 and this work
myc-Sc[1-163]	Deletion of aa 164-345	Giagtzoglou 2005 and this work
myc-Sc[164-345]	Deletion of aa 1-163	Giagtzoglou 2005 and this work
myc-m7ΔW	Deletion of W186 (destroyed WRPW motif)	Giagtzoglou 2005 and this work
myc-m7KNEQ	K15→N, E22→Q (abrogates DNA binding)	Giagtzoglou 2005 and this work
myc-m8	Wild type m8	Giagtzoglou 2005, Alifragis 1997 and this work
myc-Sc[S217A]	S217→A	this work
myc-Sc[m3p]	S268→A, T270→A, S271→A (3 putative phosphooacceptors mutated)	this work
myc-Sc[T329V]	T329→V	this work
myc-Sc[m2p]	S217→A, T329→V (2 putative phosphoacceptors mutated)	this work
myc-Sc[m5p]	S217→A, S268→A, T270→A, S271→A, T329→V (5 putative phosphoacceptors mutated)	this work
myc-Sc[RQEQ]	R104→Q, R108→Q (abrogates DNA binding)	this work
myc-Sc[RQEQ, m3p]	R104→Q, R108→Q, S268→A, T270→A, S271→A	this work
myc-ScΔ[175-199]	Deletion of aa175-199[IGANNAVTQLQLCLDESSSHSSSSS]	this work
myc-Sc[1-320, m3p]	S268→A, T270→A, S271→A and deletion of aa 321-345	this work

myc-Sc[1-320,RQEQ]	R104→Q, R108→Q and deletion of aa 321-345	this work
myc-Sc[1-318]-AD1	Deletion of Scute aa 319-345 (ScTAD) and fusion of Da AD1 (aa 1-160)	this work
myc-Sc[1-318]-LH	Deletion of Scute aa 319-345 (ScTAD) and fusion of Da LH (aa265-417)	this work
myc- Sc[321-345]-DaΔTADs	Deletion of aa 1-414 of Da (which includes both Da TADs) and fusion at its N terminus of aa 321-345 of Sc (ScTAD)	this work
myc- Sc[291-345]-DaΔTADs	Deletion of aa 1-414 of Da (which includes both Da TADs) and fusion at its N terminus of aa 291-345 of Sc (which includes ScTAD)	this work
myc- Sc[261-345]-DaΔTADs	Deletion of aa 1-414 of Da (which includes both Da TADs) and fusion at its N terminus of aa 261-345 of Sc (which includes ScTAD and SPTS motif)	this work

His tagged constructs in Ract vector[A]		
His-DaΔAD1	Deletion of aa 1-264	Zarifi 2012 and this work
His-DaΔLH	Deletion of aa 211-463	Zarifi 2012 and this work
His-DaΔTADs	Deletion of aa 1-414	Zarifi 2012 and this work
His-m7	Wild type	Zarifi 2012 and this work
His-m7ΔW	Deletion of W186 (destroyed WRPW motif)	Giagtzoglou 2005 and this work
His-m7[K72R]	K72→R	this work
His-m7[m3K]	K51→R, K54→R, K72→R (3 lysines mutated)	this work
His-m7[m2K_mut] (see Note B)	K51→R, K54→R, deletion of W186 and extension of C terminus as undelined here: WRPCRHASYSMHTHILLLIR[B] (two lysines mutated plus 3' deletion)	this work

Note A: His-m7variants have the following sequence at the N terminus (MRGSHHHHHHGMASSKF), while His-Da variants have the following sequence at their N terminus (MRGSHHHHHHGMASSRG).
Note B: Due to a cloning-induced mutation a part of the end of the m7[m2K_mut] coding sequence was deleted (underlined) (TGG/ CGG/ CCC/ TGG/ TAA CTC GAC CTG CAG GCATG CAA…) causing a frameshift.

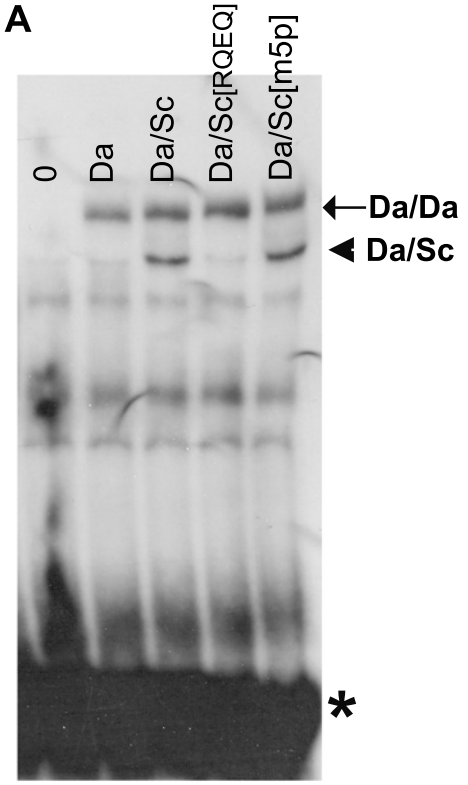
	Primers used for mutagenesis	Restriction Site
Scute (R104→Q, R108→Q)	GGTCCAAAGGCAAAATGCTCGTCAACGAAAT	HincII appears
Scute (S268→A, T270→A, S271→A)	CCACGCGCCAGCAGCATCATTCAACTCGAGCAT	XhoI appears
Scute (T329→V)	TTCAGCTGGACGAGGAGGACTGCGTCCCCG	PvuII appears
Scute (S217→A)	CTCTGTCGCTCCTCTGCAGCAACAGC	PstI appears
m7 (K51→R, K54→R)	GCGATGCCAGATTCGAGAGGGCC	Destroys ApoI and Sau96I sites of wt m7
m7 (K72→R)	GAAAGAGTCTAGGAAGCATG	Destroys DdeI of wt m7
Green highlights show the point mutations of each variant and underlined is the restriction site.		

Construction of 3x myc epitope in pBS vector

Double stranded oligonucleotide containing one repeat of myc epitope was prepared by
annealing the two complementary oligonucleotides
5'-AATTGACCATGGAGCAGAAGCTGATCAGCGAGGAGGACCTGG-3' and
5'-AATTCCAGGTCCTCCTCGCTGATCAGCTTCTGCTCCATGGTC-3'.

3xmyc epitope containing three direct tandem repeat copies of the above double-stranded oligonucleotide was constructed by annealing, ligation, and digestion with EcoRI and MfeI, selection of the tandem of three copies in an agarose gel, and subcloning into pBluescript in the EcoRI site.
The appended aminoacid sequence is $(MEQKLISEEDLELT)_2$MEQKLISEEDLEF.

Further details on all constructs are available upon request.


A
0
Da
Da/Sc
Da/Sc[RQEQ]
Da/Sc[m5p]
Da/Da
Da/Sc
*

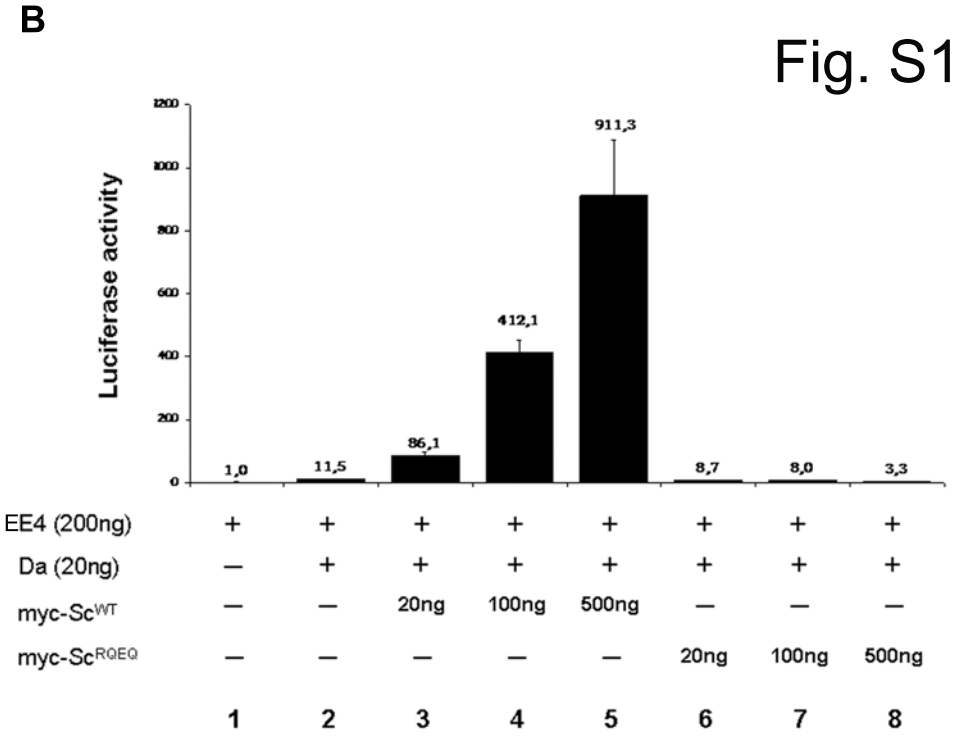

B
Luciferase activity
1200
1000
800
600
400
200
0
1,0
11,5
86,1
412,1
911,3
8,7
8,0
3,3
EE4 (200ng) + + + + + + + +
Da (20ng) — + + + + + + +
myc-Sc^WT — — 20ng 100ng 500ng — — —
myc-Sc^RQEQ — — — — — 20ng 100ng 500ng
1 2 3 4 5 6 7 8

Fig. S1

Fig. S2

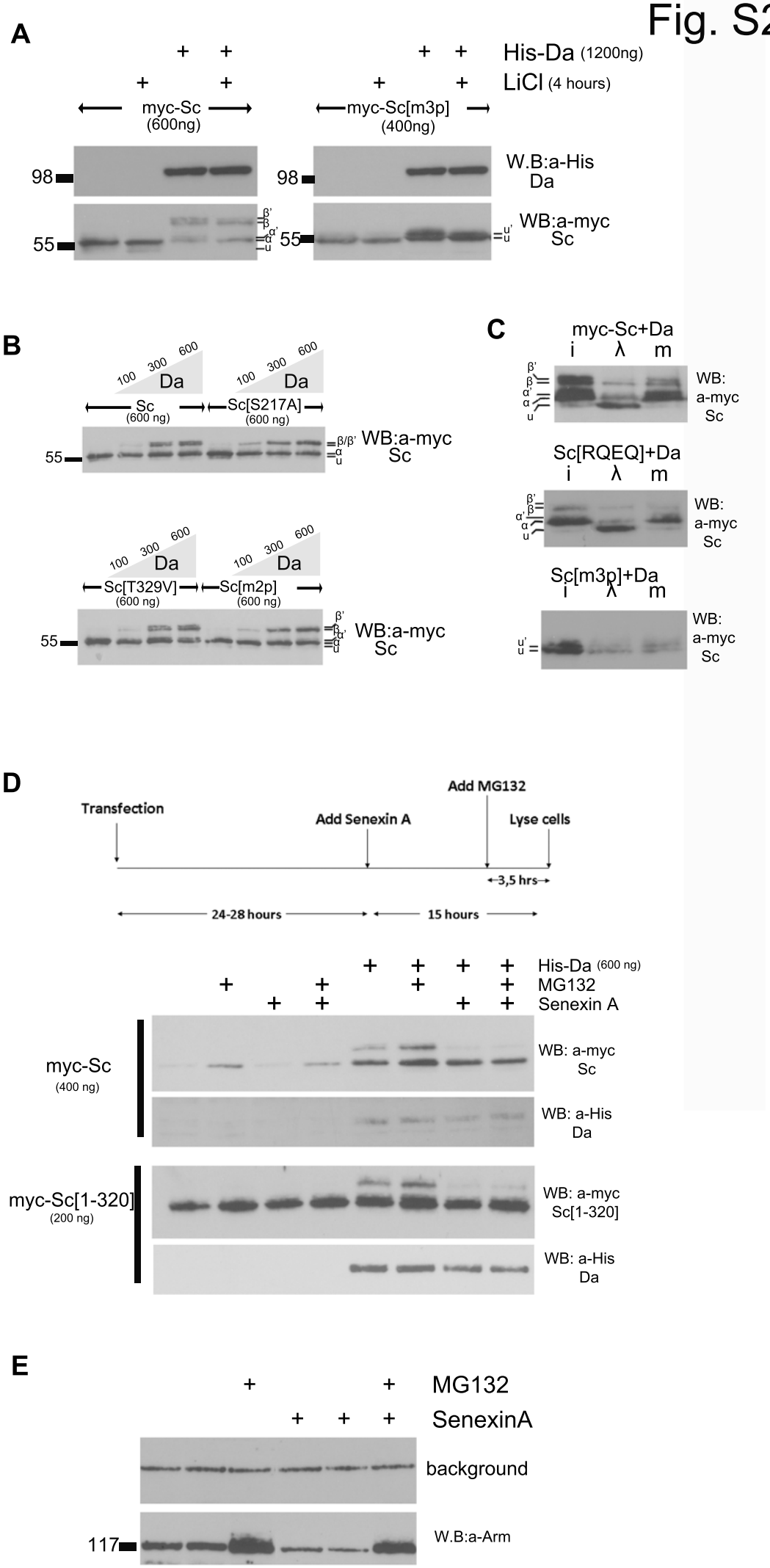
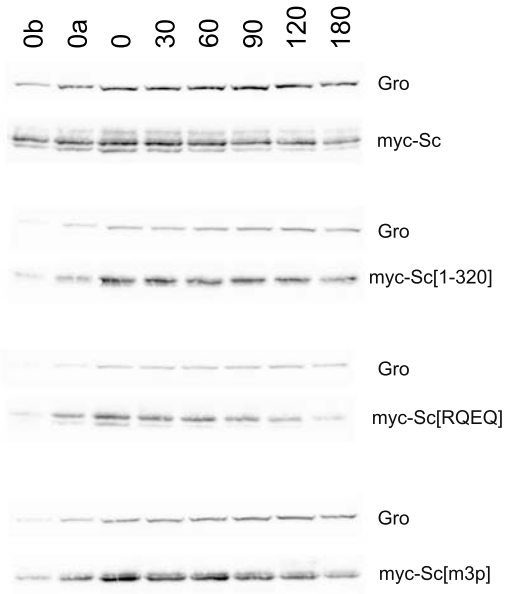
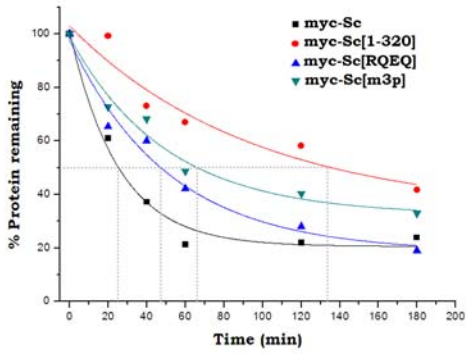

A
His-Da (1200ng)
LiCl (4 hours)
myc-Sc (600ng)
myc-Sc[m3p] (400ng)
98
55
W.B:a-His Da
WB:a-myc Sc
B
100 300 600
Da
Sc (600 ng)
Sc[S217A] (600 ng)
Sc[T329V] (600 ng)
Sc[m2p] (600 ng)
WB:a-myc Sc
C
myc-Sc+Da
Sc[RQEQ]+Da
Sc[m3p]+Da
i λ m
WB: a-myc Sc
D
Transfection
Add Senexin A
Add MG132
Lyse cells
3,5 hrs
24-28 hours
15 hours
His-Da (600 ng)
MG132
Senexin A
myc-Sc (400 ng)
myc-Sc[1-320] (200 ng)
WB: a-myc Sc
WB: a-His Da
WB: a-myc Sc[1-320]
E
MG132
SenexinA
background
117
W.B:a-Arm

Fig. S3

A


0b
0a
0
30
60
90
120
180
Gro
myc-Sc
Gro
myc-Sc[1-320]
Gro
myc-Sc[RQEQ]
Gro
myc-Sc[m3p]

B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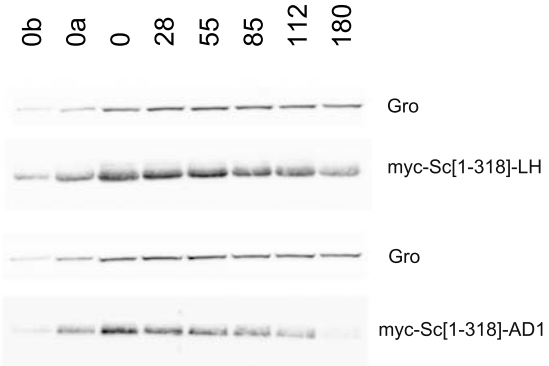
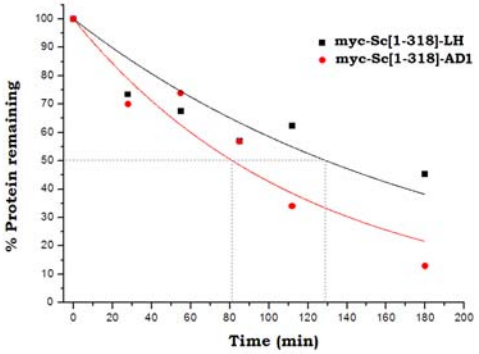
myc-Sc[1-320]
myc-Sc[RQEQ]
myc-Sc[m3p]
% Protein remaining
Time (min)

C


0b
0a
0
28
55
85
112
180
Gro
myc-Sc[1-318]-LH
Gro
myc-Sc[1-318]-AD1

D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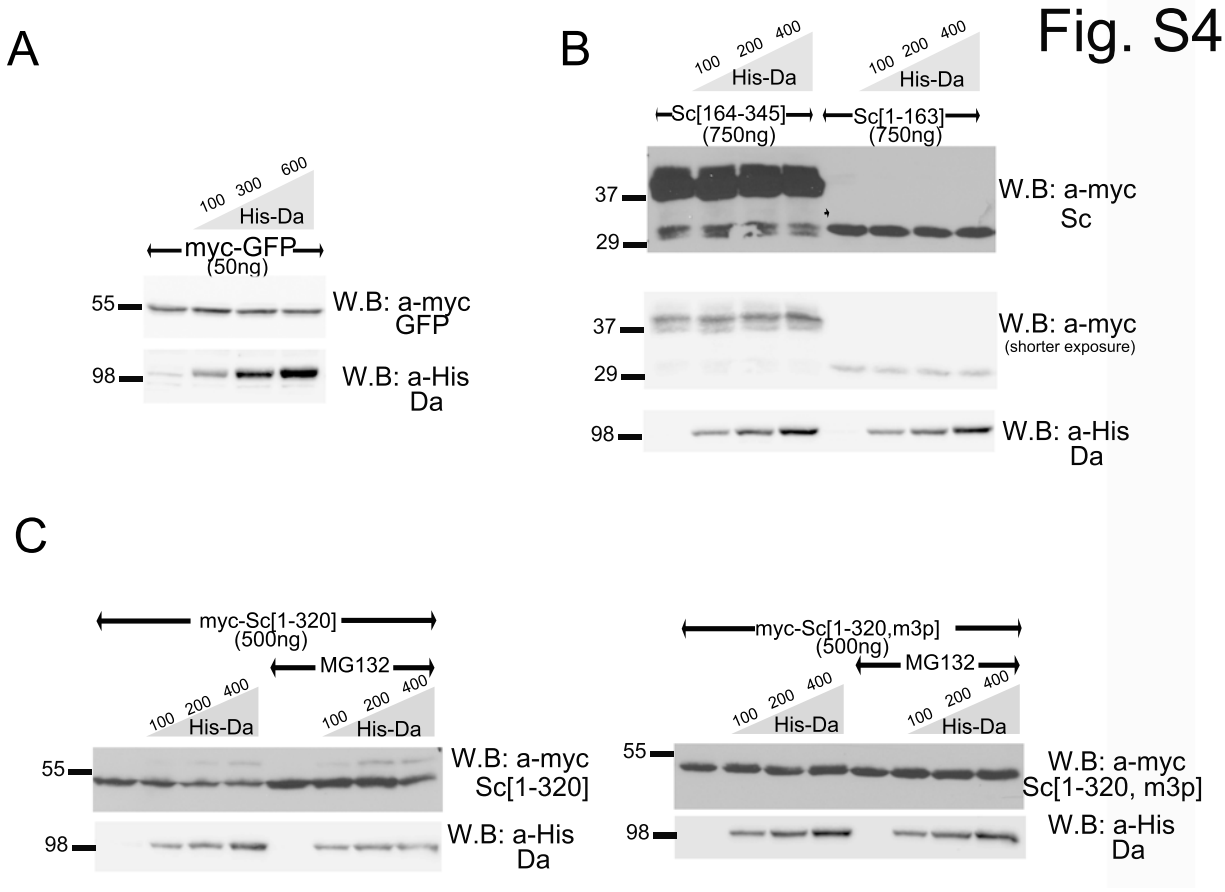
myc-Sc[1-318]-AD1
% Protein remaining
Time (min)

Fig. S4


A
100 300 600
His-Da
myc-GFP
(50ng)
55
W.B: a-myc
GFP
98
W.B: a-His
Da
B
100 200 400
His-Da
100 200 400
His-Da
Sc[164-345]
(750ng)
Sc[1-163]
(750ng)
37
29
W.B: a-myc
Sc
37
29
W.B: a-myc
(shorter exposure)
98
W.B: a-His
Da
C
myc-Sc[1-320]
(500ng)
MG132
100 200 400
His-Da
100 200 400
His-Da
55
W.B: a-myc
Sc[1-320]
98
W.B: a-His
Da
myc-Sc[1-320,m3p]
(500ng)
MG132
100 200 400
His-Da
100 200 400
His-Da
55
W.B: a-myc
Sc[1-320, m3p]
98
W.B: a-His
Da

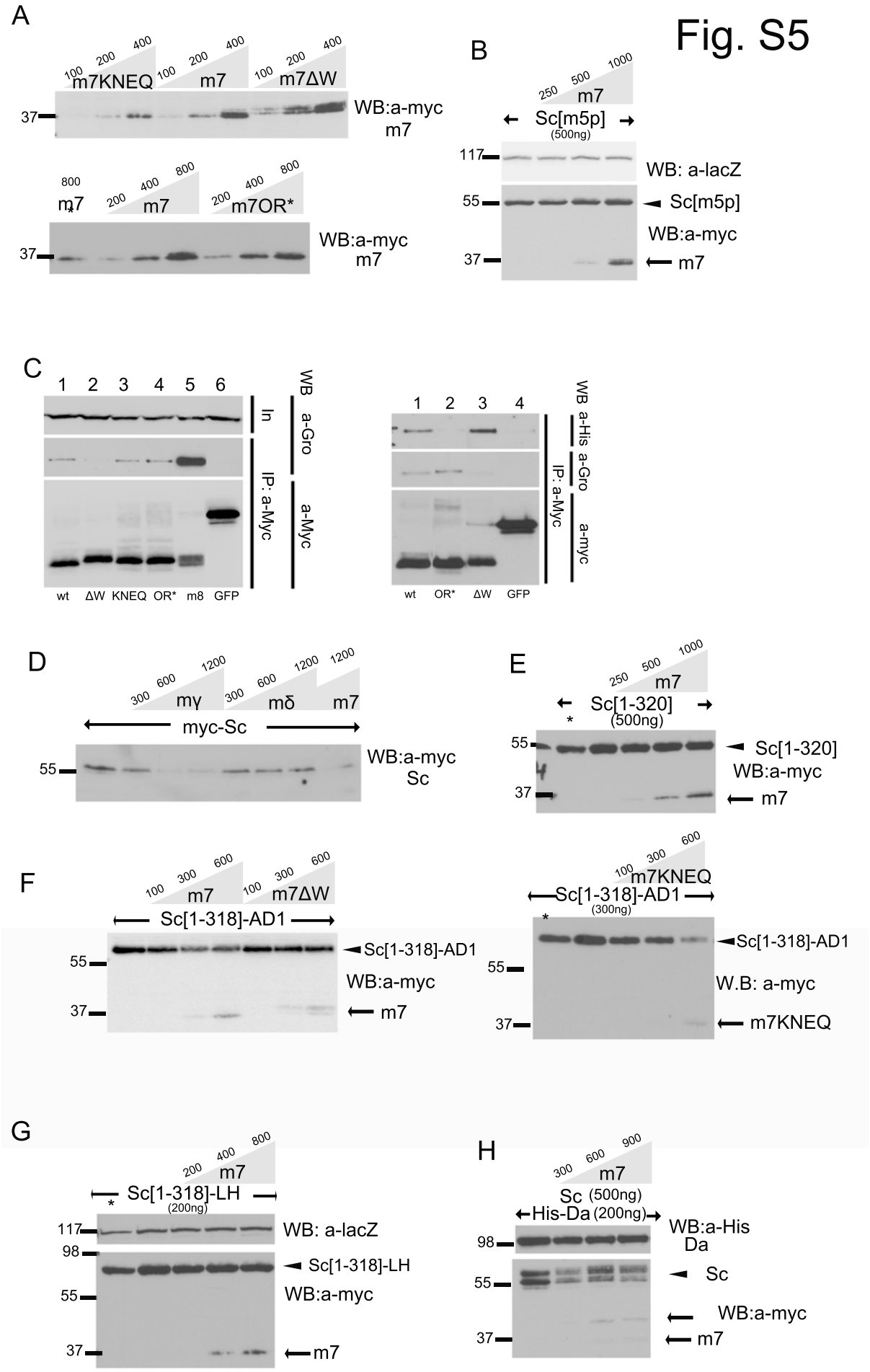


Fig. S5
A
m7KNEQ
m7
m7ΔW
WB:a-myc
m7
m7*
m7OR*
B
m7
Sc[m5p]
(500ng)
WB: a-lacZ
Sc[m5p]
WB:a-myc
m7
C
In
IP: a-Myc
WB a-Gro
a-Myc
wt ΔW KNEQ OR* m8 GFP
WB a-His
a-Gro
a-myc
wt OR* ΔW GFP
D
mγ
mδ
m7
myc-Sc
WB:a-myc
Sc
E
m7
Sc[1-320]
(500ng)
Sc[1-320]
WB:a-myc
m7
F
m7
m7ΔW
Sc[1-318]-AD1
Sc[1-318]-AD1
WB:a-myc
m7
m7KNEQ
Sc[1-318]-AD1
(300ng)
Sc[1-318]-AD1
W.B: a-myc
m7KNEQ
G
m7
Sc[1-318]-LH
(200ng)
WB: a-lacZ
Sc[1-318]-LH
WB:a-myc
m7
H
m7
Sc (500ng)
His-Da (200ng)
WB:a-His
Da
Sc
WB:a-myc
m7

Fig. S6

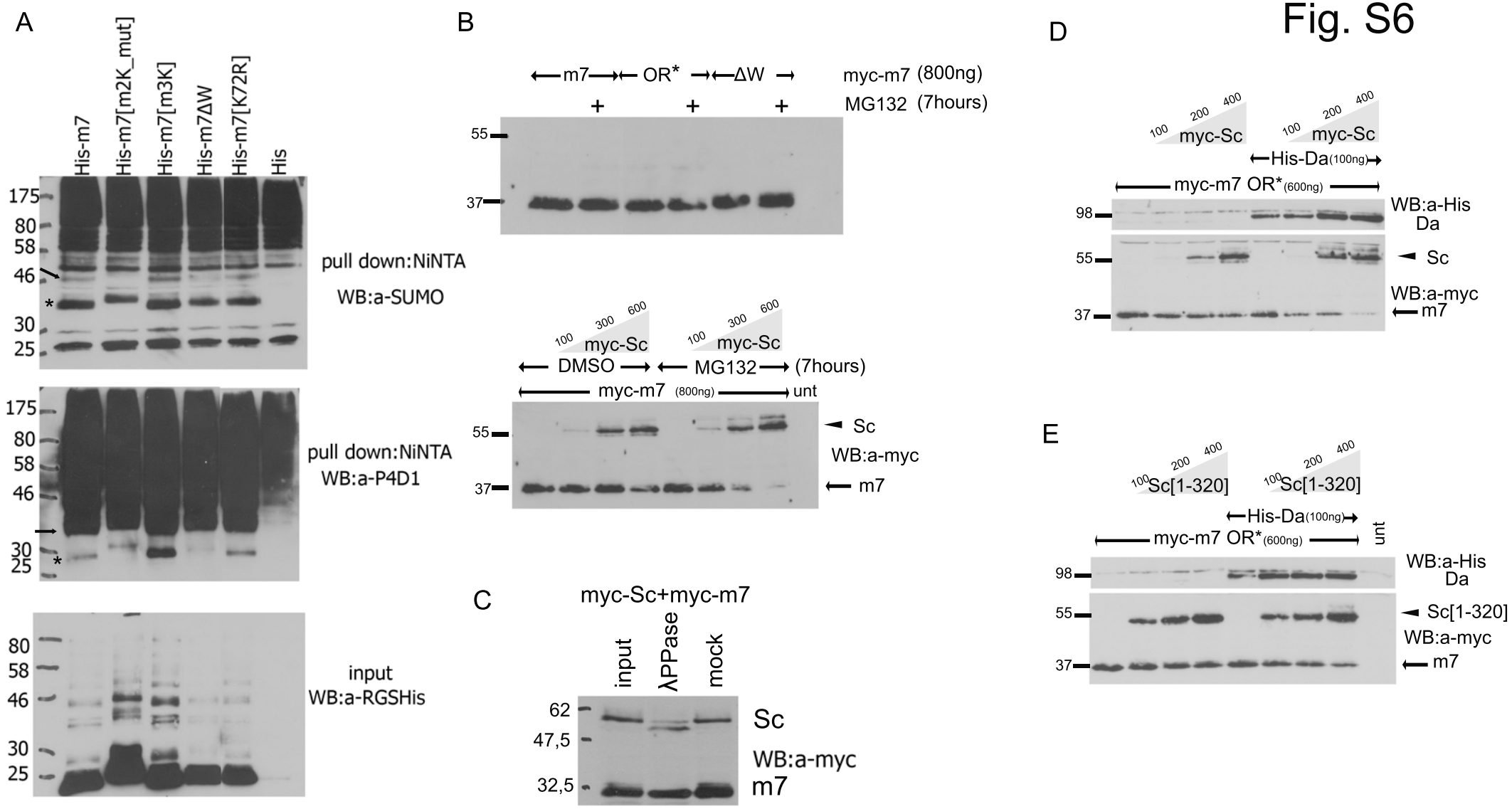
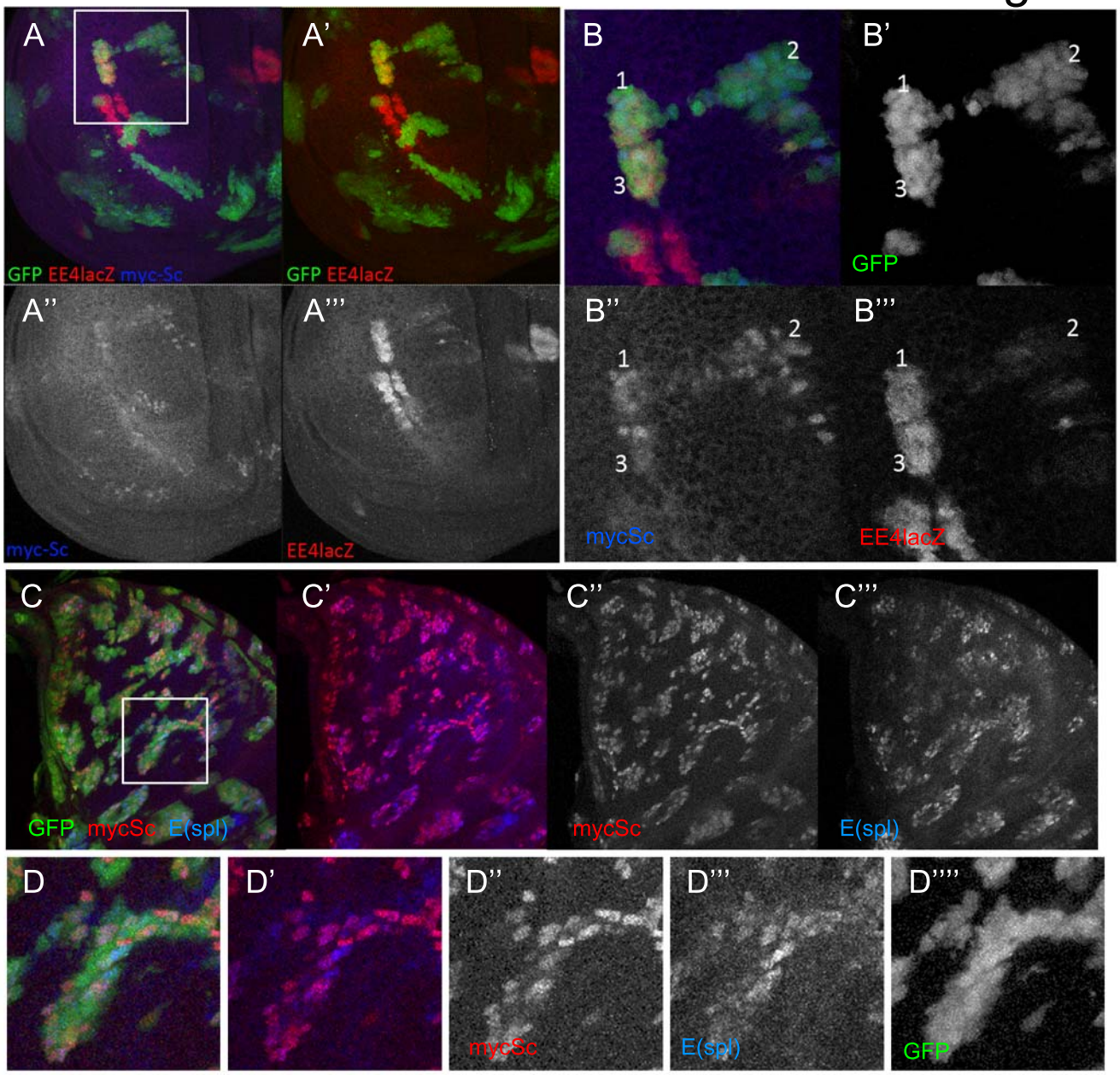

A
His-m7
His-m7[m2K_mut]
His-m7[m3K]
His-m7ΔW
His-m7[K72R]
His
175
80
58
46
30
25
pull down:NiNTA
WB:a-SUMO
pull down:NiNTA
WB:a-P4D1
input
WB:a-RGSHis
B
m7
OR*
ΔW
myc-m7 (800ng)
MG132 (7hours)
55
37
100
300
600
myc-Sc
DMSO
MG132
(7hours)
myc-m7 (800ng)
unt
Sc
WB:a-myc
m7
C
myc-Sc+myc-m7
input
λPPase
mock
62
47,5
32,5
Sc
WB:a-myc
m7
D
100
200
400
myc-Sc
His-Da(100ng)
myc-m7 OR*(600ng)
98
WB:a-His
Da
Sc
WB:a-myc
m7
E
Sc[1-320]
His-Da(100ng)
myc-m7 OR*(600ng)
unt
WB:a-His
Da
Sc[1-320]
WB:a-myc
m7

Fig. S7


A
A'
B
B'
1
2
3
GFP EE4lacZ myc-Sc
GFP EE4lacZ
GFP
A''
A'''
B''
B'''
myc-Sc
EE4lacZ
mycSc
EE4lacZ
C
C'
C''
C'''
GFP mycSc E(spl)
mycSc
E(spl)
D
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