Direct role for the *Drosophila* GIGYF protein in 4EHP-mediated mRNA repression

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Supplementary Material

Supplementary Table S1. Mutants and constructs used in this study

Protein	Name of the construct	Fragments / mutations	Binding site / motif
***Dm* 4EHP** (isoform 1) CG33100	4EHP	Full length (1–223)	
	D*	W85A	Dorsal surface
	L*	V75A, L91A	Lateral surface
	S*	R93P, E139L	Specific surface
	CAP*	W114A	Cap-binding pocket
***Dm* GIGYF** (isoform G) CG11148	GIGYF	Full length (1–1574)	
	N-term region	1–640	
	C-term region	641–1574	
	C*	Y58A, Y60A, M65A	Canonical
	ΔMBM	Δ331-374	Me31B binding motif
	GYF*	Y571A, F582A, W590A, F596A	GYF domain
	C*+GYF*	Y58A, Y60A, M65A, Y571A, F582A, W590A, F596A	Canonical + GYF domain
	ΔMBM+GYF*	Δ331-374, Y571A, F582A, W590A, F596A	Me31B binding motif + GYF domain
	C+ΔMBM+GYF*	Y58A, Y60A, M65A, Y571A, Δ331-374, F582A, W590A, F596A	Canonical + Me31B binding motif + GYF domain
	GYF	GYF domain (553-621)	
***Dm* HPat** CG5208	HPat	Full length (1–968)	
	PPGF*	P286A, P287A, F289A, P328A, P329A, F331A	GYF-domain binding mutant
	P-reg	P-rich region (57-499)	
***Dm* DCP1** CG11183	GSSG	T70G, N71S, N72S, T73G	NR-loop mutant

Supplementary Table S2. Antibodies used in this study.

Antibody	Source	Catalog Number	Dilution	Monoclonal/ Polyclonal
Anti-HA-HRP (Western blot)	Roche	12 013 819 001	1:5,000	Monoclonal
Anti-HA (Immunoprecipitation)	Covance	MMS-101P	1:1,000	
Anti-GFP	In house		IP	Polyclonal
Anti-GFP	Roche	11814460001	1:2,000	Monoclonal
Anti-rabbit-HRP	GE Healthcare	NA934V	1:10,000	Polyclonal
Anti-mouse-HRP	GE Healthcare	RPN4201	1:10,000	
Anti-V5	QED Bioscience Inc.	18870	1:5,000	
Anti-V5	LSBio LifeSpan BioSciences, Inc.	LS-C57305	1:5,000	Monoclonal
Anti- *Dm* Me31B	In house		1:3,000	Polyclonal
Anti- *Dm* HPat	In house		1:3,000	
Anti-*Dm* PABP	In house		1:5,000	
Anti-*Dm* NOT3	In house		1:3,000	
Anti-*Dm* DCP1	In house		1:2,000	
Anti-*Hs* CNOT1	In house		1:1,000	
Anti-*Hs* CNOT3	Abcam	ab55681	1:2,000	Monoclonal
Anti-*Hs* CNOT2	Bethyl Laboratories	A302-562A	1:2,000	Polyclonal
Anti-*Hs* DDX6	Abcam	ab95030	1:2,000	

Supplementary Table S3. Nomenclature of orthologous genes in *Dm* and human

Dm	**Human**
GIGYF	GIGYF1/2
GW182	TNRC6A/B/C
Tis11	TTP
HPat	PatL1
Me31B	DDX6
NOT1	CNOT1
NOT2	CNOT2
NOT3	CNOT3
Tral	LSM14B
DCP1	DCP1A/B
Ge-1/EDC4	EDC4
POP2	CNOT7
CCR4	CNOT6L
CAF40	CNOT9
XRN1	XRN1

Supplementary Figure legends

Supplementary Figure S1. *Dm* GIGYF interacts with 4EHP. Related to Figure 1.

(A) Cartoon representation of the structure of *Hs* 4EHP (in yellow) bound to *Hs* GIGYF2 proteins (in blue) according to Peter et al. 2017 (1). The dorsal, lateral and 4EHP-specific surfaces or the canonical (C), noncanonical (NC) and auxiliary (A) binding motifs of GIGYF2 are indicated in the figure. N and C represent Amino- and Carboxyl-termini, respectively.

(B) Tethering assay using the F-Luc-V5 (without BoxB elements) reporter and the indicated λN-HA-4EHP proteins in *Dm* S2 cells. A plasmid expressing R-Luc-A_{90}-HhR served as a transfection control. The F-Luc activity was normalized to that of the R-Luc transfection control and set to 100% in cells expressing the λN-HA peptide. Bars represent the mean values and error bars denote the standard deviation from at least three independent experiments.

(C, D) Representative F-Luc (C) and R-Luc (D) luminescence signal values detected in the experimental conditions described in B. Bars represent the mean values and error bars denote the standard deviation from at least three technical replicates.

(E) Western blot analysis showing the expression of the λN-HA-4EHP proteins used in the tethering assay described in panel (B).

(F) The interaction of GFP-4EHP with HA-Brat and HA-GIGYF was assayed by anti-GFP immunoprecipitation. GFP-F-Luc-V5 was used as a negative control. The input (3% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins) were analyzed by western blotting using anti-GFP and anti-HA antibodies.

(G) Immunoprecipitation assay to analyze binding of GFP-Bicoid to HA-4EHP. GFP-GIGYF was used as a positive control and GFP-F-Luc-V5 as a negative control for the interaction with 4EHP. Proteins were immunoprecipitated using anti-GFP antibodies. The input (3% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (15% for GFP-tagged

proteins and 30% for HA-tagged proteins) were analyzed by western blotting using anti-GFP and anti-HA antibodies.

(H) Western blot showing the interaction of HA-eIF4E and HA-4EHP with GFP-tagged-*Dm* 4E-T (CG32016). The proteins were immunoprecipitated using anti-HA antibodies. HA-MBP served as a negative control. The inputs (1% for GFP-tagged proteins and 1.5% for HA-tagged proteins) and bound fractions (30% for GFP-tagged proteins and 25% for HA-tagged proteins) were analyzed by western blotting using anti-GFP and anti-HA antibodies.

Supplementary Figure S2. Conservation of molecular features in *Dm* 4EHP. Related to Figure 1.

(A) Sequence alignment of 4EHP orthologous proteins from *Drosophila melanogaster* (*Dm*), *Homo sapiens* (*Hs*), *Mus musculus* (*Mm*) and *Danio rerio* (*Dr*). In the aligned sequences, residues with >70% similarity are shown with a light color background and conserved residues are highlighted with a darker background and printed in white. Secondary structure elements are indicated above the sequences for 4EHP and are based on the structure of the human protein [PDB: 5NVK; (1)]. Residues that were mutated in this study are indicated by open squares colored as follows: red (dorsal surface), green (lateral surface), yellow (4EHP-specific residues) and orange (cap pocket).

(B) The interaction of GFP-GIGYF with HA-4EHP [either WT or cap mutant (CAP*)] was analyzed by immunoprecipitation assay in S2 cells using anti-GFP antibodies. GFP-F-Luc-V5 served as negative control. The input (3% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (15% for GFP-tagged proteins and 20% for HA-tagged proteins) were analyzed by western blotting using anti-HA and anti-GFP antibodies.

(C) Western blot analysis of control and GIGYF-depleted cells. Due to the lack of anti-*Dm* GIGYF antibodies, we determined the efficacy and specificity of the GIGYF depletion in S2 cells with transfected HA-GIGYF plasmid and dsRNA targeting either the neomycin or the

GIGYF mRNAs. Dilutions of control cell lysates were loaded in lanes 1-3 to estimate the efficacy of the depletion. PABP was used as a loading control and F-Luc-V5 as transfection control.

Supplementary Figure S3. *Dm* GIGYF promotes mRNA decay. Related to Figure 2.

A) Tethering assay using the F-Luc-V5 (without BoxB elements) reporter and the indicated λN-HA-GIGYF proteins in *Dm* S2 cells. A plasmid expressing R-Luc-A_{90}-HhR served as a transfection control. Luciferase activity (green bars) and reporter mRNA levels (blue bars) were normalized to those of the R-Luc transfection control and set to 100% in cells expressing the λN-HA peptide. Bars represent the mean values and error bars denote the standard deviation from at least three independent experiments.

(B) Northern blot analysis of a representative tethering experiment shown in (A).

(C) Half-life experiment. Tethering assay using the F-Luc-5BoxB reporter, the R-Luc-A_{90}-HhR reporter and the indicated λN-HA-tagged proteins. Three days after transfection, cells were treated with actinomycin D and harvested at the indicated time points. Northern blot analysis was performed with samples collected at the indicated time points. *rp49* mRNA served as a loading control. The red dashed line indicates the position of the F-Luc-5BoxB mRNA decay intermediate lacking a poly(A) tail (A_0). A_n marks the position of the adenylated F-Luc reporter mRNA.

(D) Quantification of the amount of F-Luc-5BoxB mRNA present at the indicated time points following the addition of actinomycin D (n=3). The half-life of the reporter mRNA in the presence of the different proteins ($t_{1/2}$) is represented as the mean ± standard deviation.

(E) Western blot analysis showing the expression of the proteins used in the experiment depicted in Figure 2E and F.

Supplementary Figure S4. GIGYF proteins interact with components of the decapping complex. Related to Figure 3.

(A) The association of GFP-tagged EDC3 and GFP-tagged Tral with HA-tagged GIGYF and endogenous Me31B was determined by immunoprecipitation using anti-GFP antibodies. GFP-F-Luc-V5 served as negative control. The input (3.5% for GFP-tagged proteins and 1% for HA-tagged proteins and endogenous Me31B) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins and Me31B) were analyzed by western blotting using the indicated antibodies.

(B-C) Immunoprecipitation assay, using anti-GFP antibodies, showing the interaction of GFP-*Hs* PatL1 with MS2-HA-*Hs* GIGYF1 (B) and MS2-HA-*Hs* GIGYF2 (C) in HEK293T cells. V5-SBP-MBP-F-Luc-GFP was used as a negative control. The input (3% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins) were analyzed by western blotting using anti-HA and anti-GFP antibodies.

(D-E) The association of HA- or GFP-tagged GIGYF with GFP- or HA-tagged components of the decapping complex (EDC4 and DCP1) and endogenous Me31B was determined by immunoprecipitation using anti-GFP. The input (1% for HA-tagged proteins, 3% for GFP-tagged proteins and endogenous Me31B) and bound fractions (30% for HA-tagged proteins and Me31B, 15% for GFP-tagged proteins) were analyzed by western blotting using the indicated antibodies.

(F-G) Western blot showing the lack of interaction between DCP2-V5 or XRN1-V5 and HA-GIGYF in S2 cells after an immunoprecipitation assay. Nevertheless, XRN1 strongly bound to endogenous DCP1. The immunoprecipitation was carried out using an anti-V5 antibody. The input (2% for V5-tagged proteins and 1% for HA-tagged proteins and endogenous DCP1) and bound fractions (20% for V5-tagged proteins and 30% for HA-tagged proteins and endogenous DCP1) were analyzed by western blotting using anti-V5, anti-DCP1 and anti-HA antibodies.

(H) GST pulldown assay showing the interaction between GST-*Dm* GIGYF GYF domain (residues P553-H621) and MBP-*Dm* HPat P-rich region (P-reg; residues 57-499). MBP served as a negative control. The starting material (2%) and bound fractions (15.2%) were analysed by SDS-PAGE followed by Coomassie blue staining. The size markers (kDa) are shown to the left of the panel.

Supplementary Figure S5. GIGYF proteins interact with components of the deadenylation complex. Related to Figure 3.

(A) The association of GFP-NOT3 with HA-tagged GIGYF was determined by immunoprecipitation using anti-GFP antibodies. GFP-F-Luc-V5 served as negative control. The input (2.5% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins) were analyzed by western blotting using the indicated antibodies.

(B) Interaction between HA-GIGYF and GFP-tagged subunits of the CCR4-NOT deadenylase complex (CCR4, POP2 and NOT2) determined after immunoprecipitation using anti-GFP antibodies. The input (3.5% for GFP-tagged proteins, 1% for HA-tagged proteins and for endogenous NOT3) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins and for endogenous NOT3) were analyzed by western blotting using the indicated antibodies.

(C) Analysis of the interaction of V5-SBP-tagged *Hs* GIGYF1 and GIGYF2 with the NOT module (CNOT1, CNOT2 and CNOT3) of the CCR4-NOT complex and DDX6 in HEK293T cells. The proteins were pulled down using streptavidin-binding beads. The input (1.25% for V5-tagged proteins, 1% for endogenous CNOT1, CNOT2 and CNOT3 and endogenous DDX6) and bound fractions (2% for V5-tagged proteins, 22.5% for endogenous CNOT1, CNOT2 and CNOT3 and 7.5% for endogenous DDX6) were analyzed by western blotting using the indicated antibodies.

(D) Interaction of HA-GIGYF with the GFP-tagged subunits of the *Dm* PAN2-PAN3 deadenylation complex. Endogenous PABP is a known partner of the PAN2-PAN3 complex. The input (3.5% for GFP-tagged proteins and 1% for HA-tagged proteins and endogenous PABP) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins and endogenous PABP) were analyzed by western blotting using anti-GFP, anti-PABP and anti-HA antibodies.

(E) Immunoprecipitation assay in S2 cells, using anti-GFP antibodies, showing the absence of interaction between GFP-*Dm* CAF40 and HA-*Dm* GIGYF. GFP-MBP served as negative control. The input (3% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (20% for GFP-tagged proteins and 30% for HA-tagged proteins) were analyzed by western blotting using the indicated antibodies.

Supplementary Figure S6. *Dm* GIGYF does not interact with *Dm* Tis11, *Dm* ZNF598 and *Dm* GW182 in S2 cells. Related to Figure 3.

(A, B) Immunoprecipitation assay showing the absence of interaction between HA-GIGYF and GFP-*Dm* TTP (Tis11) or GFP-*Dm* ZNF598. Nevertheless, GFP-4EHP strongly bound to HA-GIGYF. The proteins were immunoprecipitated using anti-GFP antibodies. GFP-F-Luc-V5 served as a negative control. The input (3% for GFP-tagged proteins and 1% for HA-tagged proteins) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins) were analyzed by western blotting using anti-GFP and anti-HA antibodies.

(C) GFP-GIGYF does not interact with HA-*Dm* GW182. The proteins were immunoprecipitated using anti-GFP antibodies. GFP-F-Luc-V5 served as negative control and endogenous Me31B as a partner of GIGYF. The input (3% for GFP-tagged proteins and 1.5% for HA-tagged proteins and endogenous Me31B) and bound fractions (15% for GFP-tagged proteins and 30% for HA-tagged proteins and endogenous Me31B) were analyzed by western

blotting using anti-GFP, anti-HA and anti-Me31B antibodies. The asterisk denotes the IgG heavy chain band of the GFP antibody used to immunoprecipitate the GFP proteins.

Supplementary Figure S7. *Dm* GIGYF induces mRNA decay independently of 4EHP. Related to Figure 4.

(A) The interaction of WT or mutant (C*, GYF*, C*+GYF*) HA-*Dm* GIGYF proteins with GFP-4EHP was determined by anti-HA immunoprecipitation. The input (0.5%) and bound fractions (35%) were analyzed by western blotting using anti-GFP and anti-HA antibodies.

(B, C) S2 cells were transfected with the F-Luc-5BoxB reporter, WT or the indicated λN-HA-GIGYF mutants and the transfection control R-Luc-A_{90}-HhR plasmids. Luciferase activity (green bars) and reporter mRNA levels (blue bars) were analyzed as described in Supplementary Figure S3A. A northern blot analysis (C) of representative RNA samples is shown next to the graph.

(D-F) S2 cells were transfected with the F-Luc-V5 reporter that lacks the BoxB elements, the indicated λN-HA-GIGYF plasmids and the R-Luc-A_{90}-HhR plasmid. Samples were analyzed as described in Supplementary Figure S3A. Northern blot analysis (E) of representative RNA samples and western blot (F) showing the equivalent expression of the λN-HA-GIGYF proteins are depicted next to the graph.

Supplementary Figure S8. *Dm* GIGYF requires Me31B to downregulate mRNA expression. Related to Figure 4.

(A-C) Tethering assay using the F-Luc-5BoxB reporter and the indicated λN-HA-tagged proteins in S2 cells. Samples were analyzed as described in Supplementary Figure S3A. Northern blot analysis (B) of a representative experiment is shown next to the graph. A western blot showing the expression levels of the tethered proteins related to the experiment is shown in panel C. The *P* value (***P < 0.0005) was determined using the two-tailed Student's t test.

(D-K) S2 cells were treated with dsRNA targeting neomycin and Me31B mRNAs and transfected with a mixture of three plasmids coding for F-Luc-5BoxB, R-Luc-A_{90}-HhR and the indicated λN-HA-tagged proteins. In panels D and H, luciferase activity (green bars) and reporter mRNA levels (blue bars) were analyzed in the presence of the different tethered proteins, as described in Supplementary Figure S3A. Representative northern blot analysis are shown in panels E and I. Western blot analysis showing the similar expression of the λN-HA-GIGYF proteins in the different experimental conditions is depicted in panels F and J. The efficacy of the different knockdowns was estimated in panels G and K. Dilutions of control cell lysates were loaded in lanes 1-3 to estimate the efficacy of the depletion. PABP served as a loading control. The *P* value (***P < 0.0005) was determined using the two-tailed Student's t test to compare the different experimental conditions with WT GIGYF in control conditions.

Supplementary Figure S9. *Dm* GIGYF requires Me31B and HPat to downregulate mRNA expression. Related to Figure 4.

(A-F) S2 cells were treated with dsRNA targeting neomycin, Me31B and/or HPat mRNAs and transfected with a mixture of three plasmids coding for F-Luc-5BoxB, R-Luc-A_{90}-HhR and λN-HA-GIGYF. In panels A and D, luciferase activity (green bars) and reporter mRNA levels (blue bars) were analyzed in the presence of the different tethered proteins, as described in Supplementary Figure S3A. Representative northern blot analysis are shown in panels B and E. The efficacy of the different knockdowns was estimated in panels C and F. Dilutions of control cell lysates were loaded in lanes 1-3 to estimate the efficacy of the depletion. PABP served as a loading control. The *P* value (***P < 0.0005) was determined using the two-tailed Student's t test.

Supplementary Figure S10. *Dm* GIGYF requires Me31B and HPat to repress translation. Related to Figure 4.

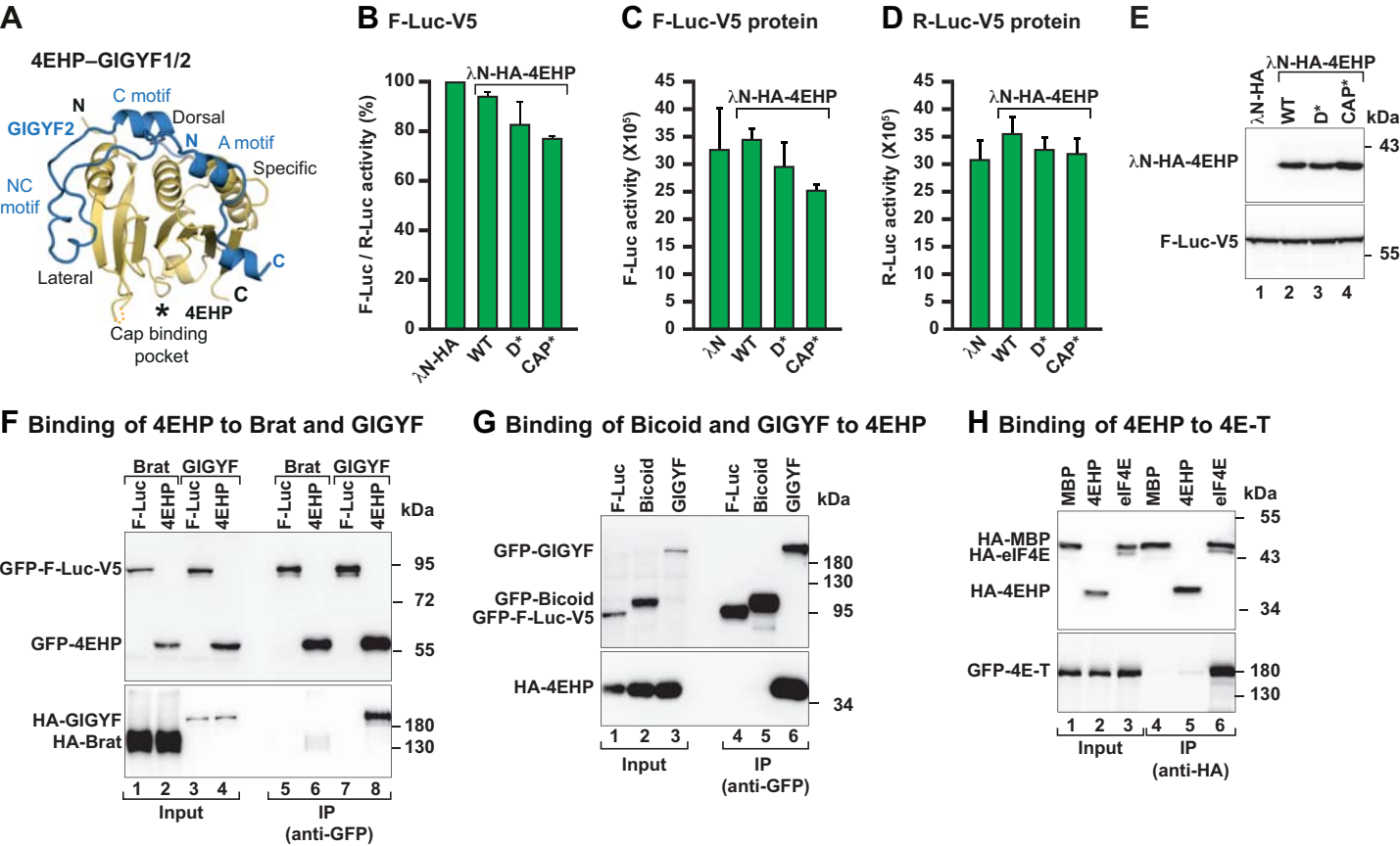
(A-B) Tethering assay using the F-Luc-5BoxB-A_{96}-HhR reporter and the indicated λN-HA-tagged proteins in S2 cells. Samples were analyzed as described in Supplementary Figure S3A. A western blot showing the expression levels of the tethered proteins related to the experiment is shown in panel B.

(C-H) S2 cells were treated with dsRNA targeting neomycin, Me31B or HPat mRNAs and transfected with a mixture of three plasmids coding for F-Luc-5BoxB-A_{96}-HhR, R-Luc-A_{90}-HhR and the indicated λN-HA-tagged proteins. Luciferase activity (green bars) and reporter mRNA levels (blue bars) were analyzed in the presence of the different tethered proteins, as described in Supplementary Figure S3A. Representative northern blots are shown in panels D and H. A western blot showing the expression levels of the tethered proteins related to the experiment described in C is shown in panel E. The efficacy of the Me31B knockdown was estimated in panel F. Dilutions of control cell lysates were loaded in lanes 1-3 to estimate the efficacy of the depletion. PABP served as a loading control. The *P* value (***P < 0.0005) was determined using the two-tailed Student's t test and is relative to the GIGYF WT protein in Ctrl KD conditions.

References

1. Peter, D., Weber, R., Sandmeir, F., Wohlbold, L., Helms, S., Bawankar, P., Valkov, E., Igreja, C. and Izaurralde, E. (2017) GIGYF1/2 proteins use auxiliary sequences to selectively bind to 4EHP and repress target mRNA expression. *Genes Dev*, **31**, 1147-1161.

Ruscica *et al.* Fig. S1



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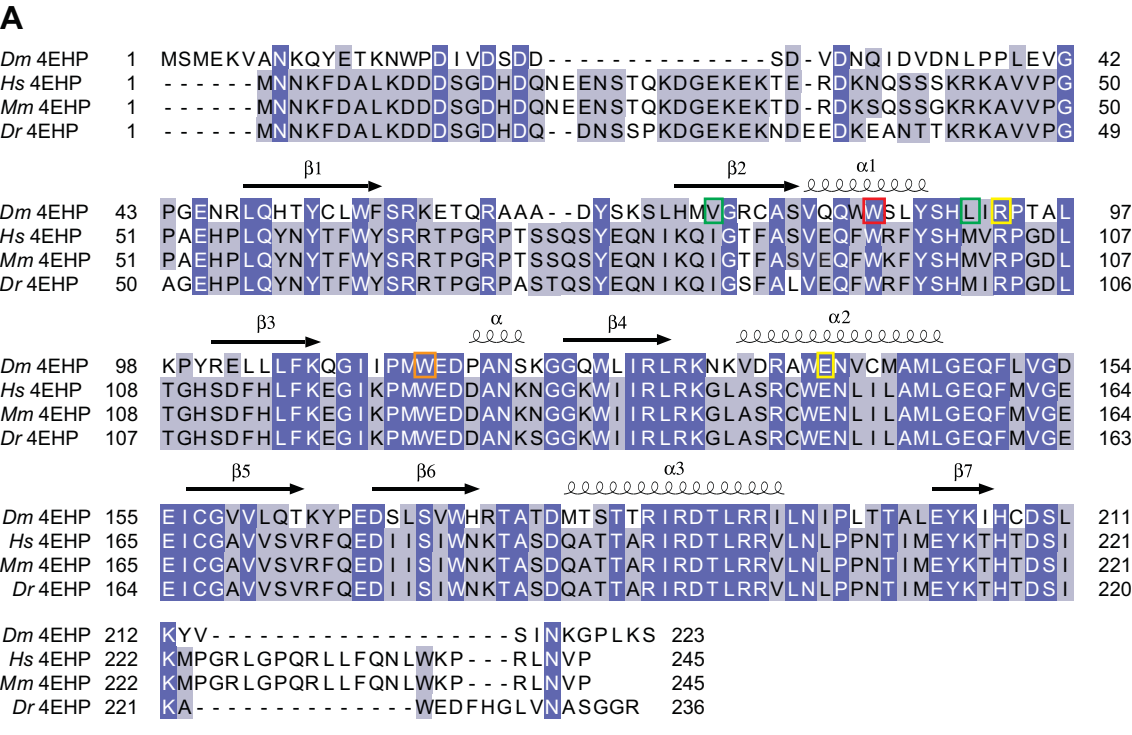

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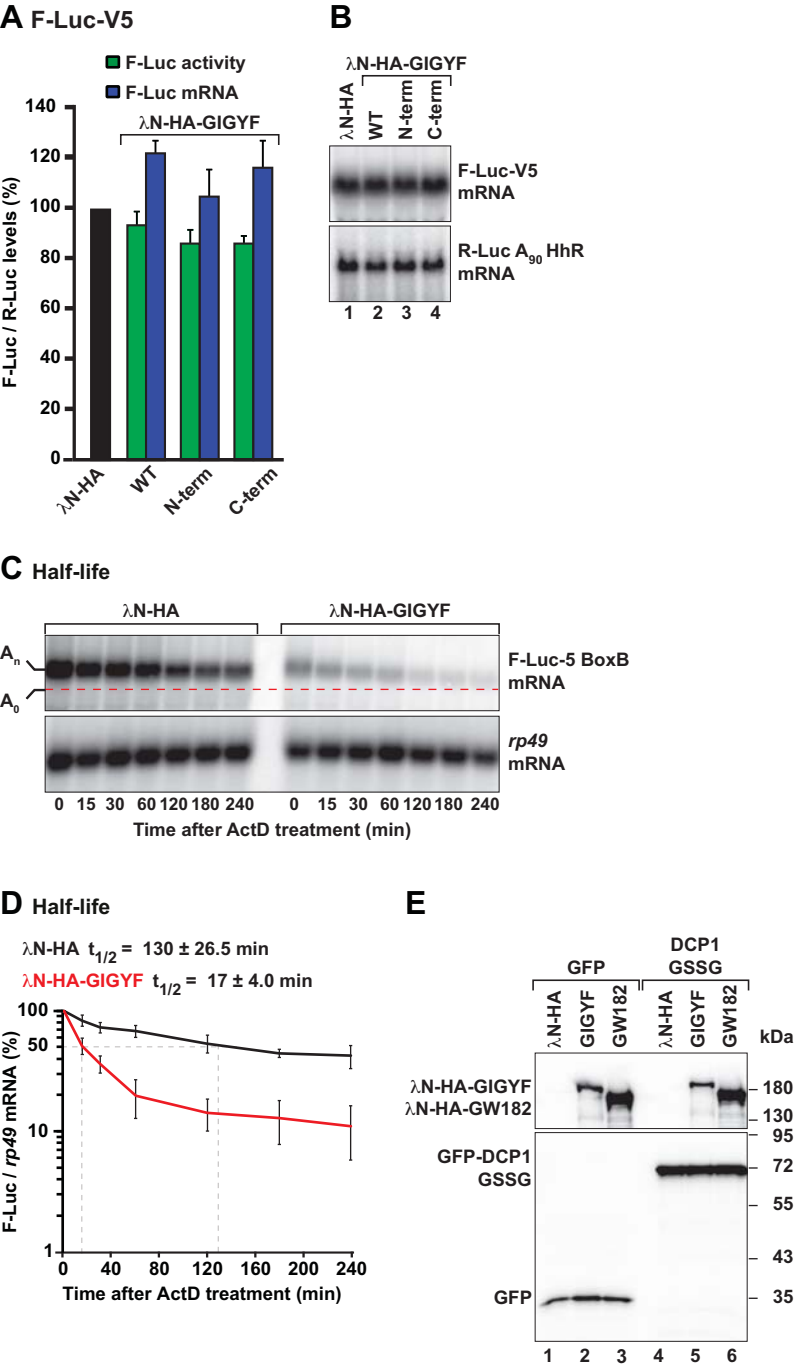

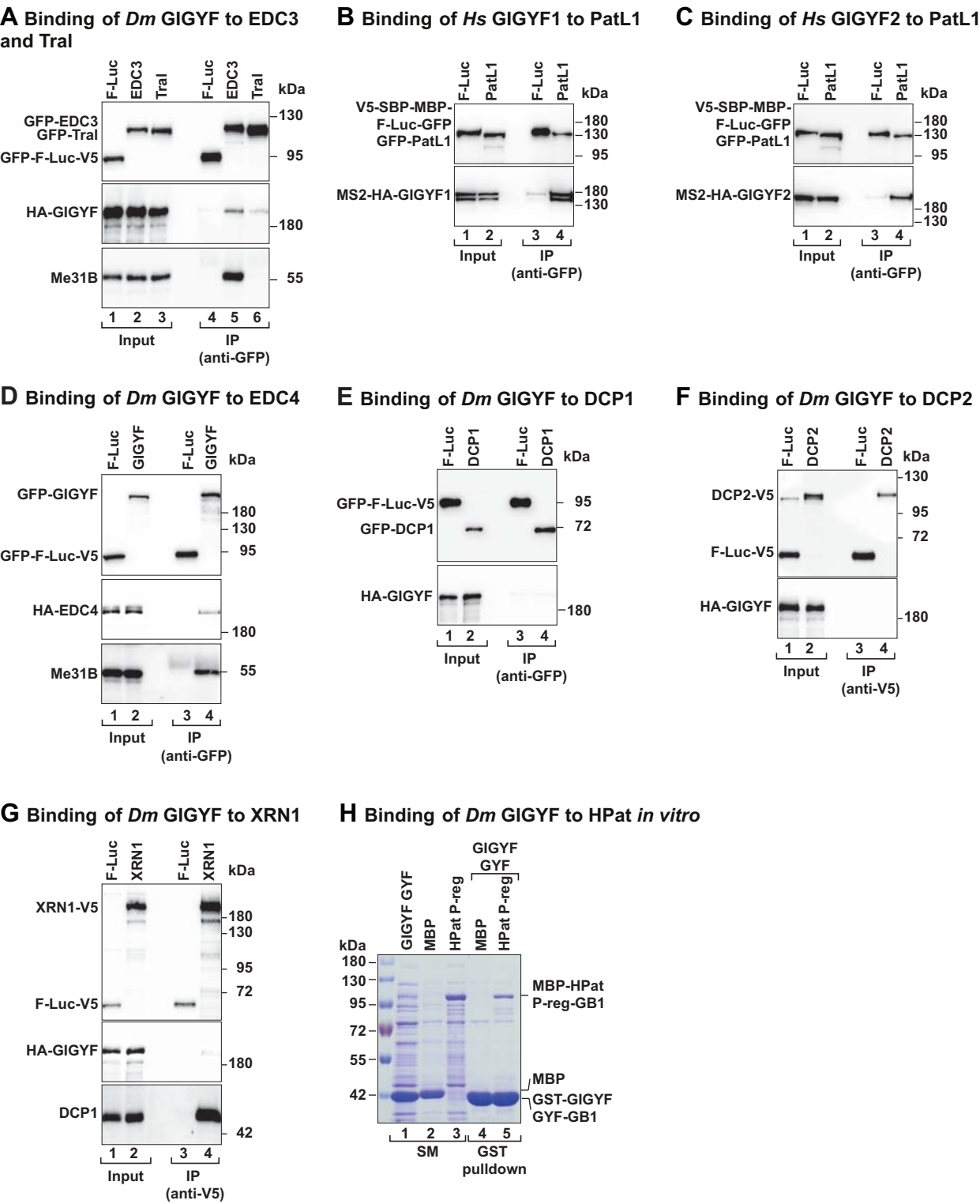
A Binding of Dm GIGYF to EDC3 and Tral
F-Luc
EDC3
Tral
kDa
GFP-EDC3
GFP-Tral
GFP-F-Luc-V5
HA-GIGYF
Me31B
130
95
180
55
1 2 3 4 5 6
Input
IP (anti-GFP)
B Binding of Hs GIGYF1 to PatL1
PatL1
V5-SBP-MBP-F-Luc-GFP
GFP-PatL1
MS2-HA-GIGYF1
180
130
95
1 2 3 4
Input
IP (anti-GFP)
C Binding of Hs GIGYF2 to PatL1
MS2-HA-GIGYF2
D Binding of Dm GIGYF to EDC4
GIGYF
GFP-GIGYF
GFP-F-Luc-V5
HA-EDC4
Me31B
72
IP (anti-GFP)
E Binding of Dm GIGYF to DCP1
DCP1
GFP-F-Luc-V5
GFP-DCP1
HA-GIGYF
F Binding of Dm GIGYF to DCP2
DCP2
DCP2-V5
F-Luc-V5
HA-GIGYF
IP (anti-V5)
G Binding of Dm GIGYF to XRN1
XRN1
XRN1-V5
F-Luc-V5
HA-GIGYF
DCP1
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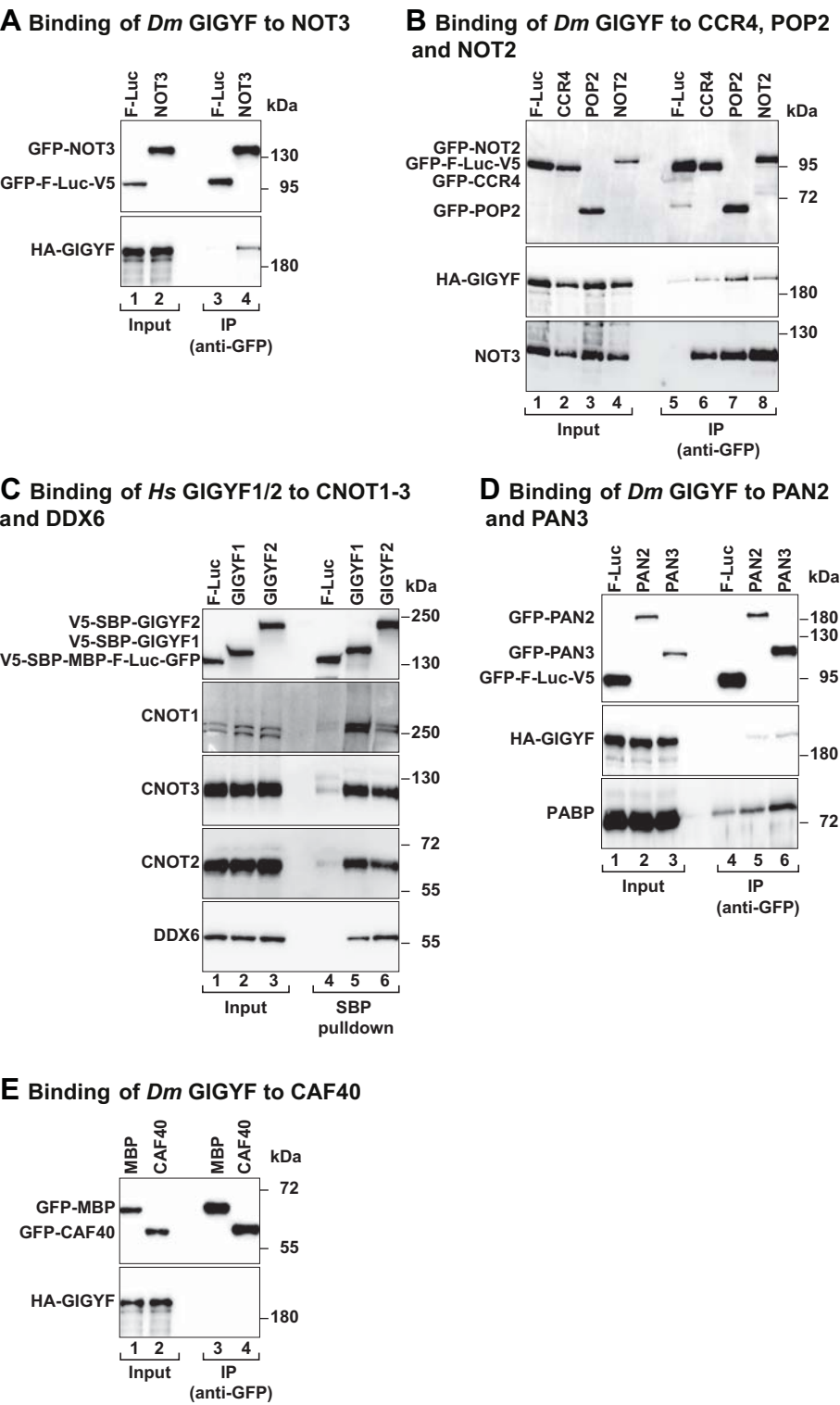
IP (anti-V5)
H Binding of Dm GIGYF to HPat in vitro
GIGYF GYF
GYF
MBP
HPat P-reg
MBP-HPat P-reg-GB1
MBP
GST-GIGYF GYF-GB1
1 2 3 4 5
SM
GST pulldown

Ruscica *et al.* Fig. S5



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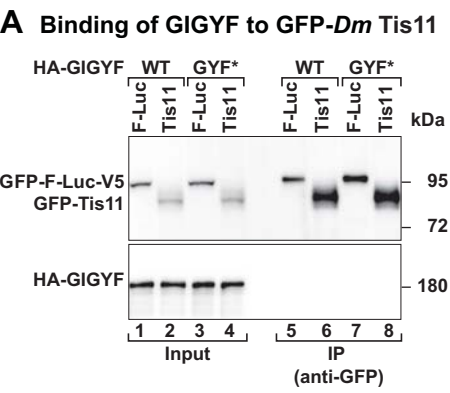
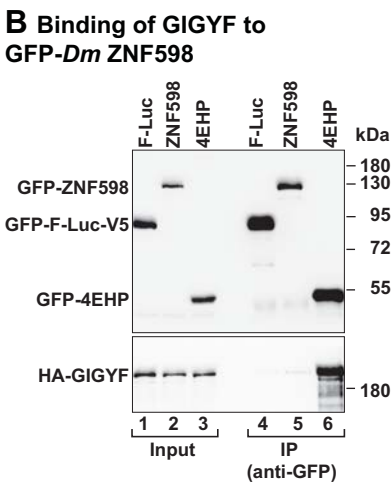
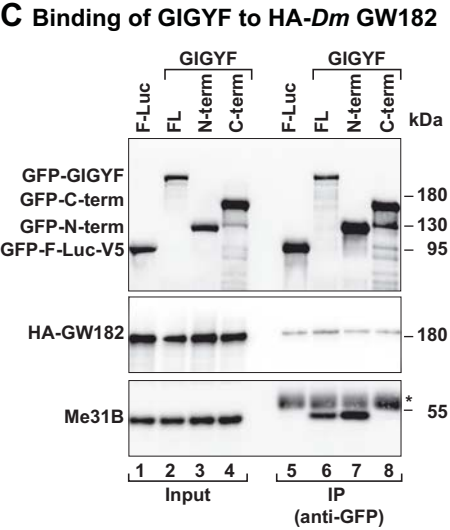
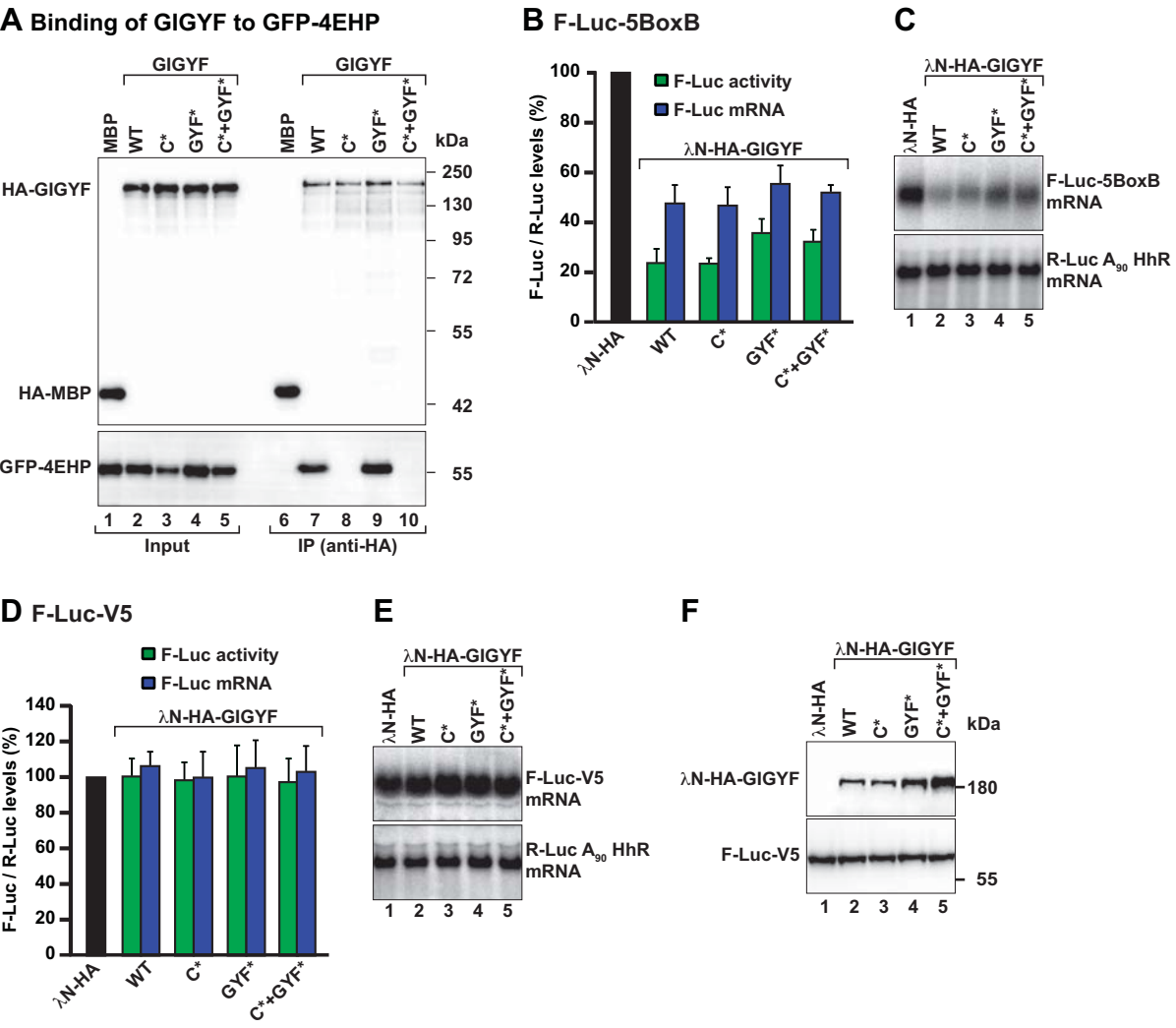






Ruscica *et al.* Fig. S7



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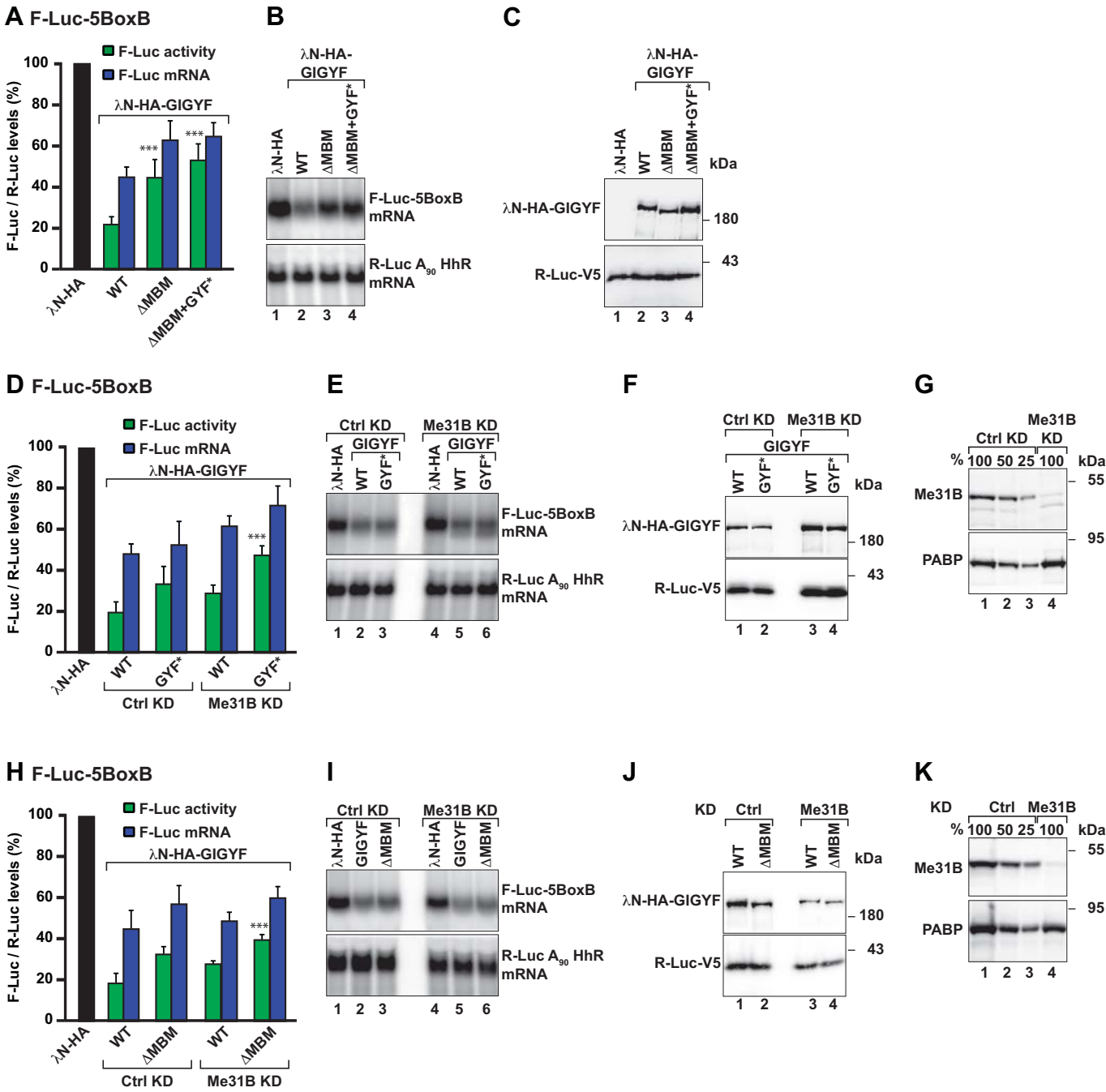

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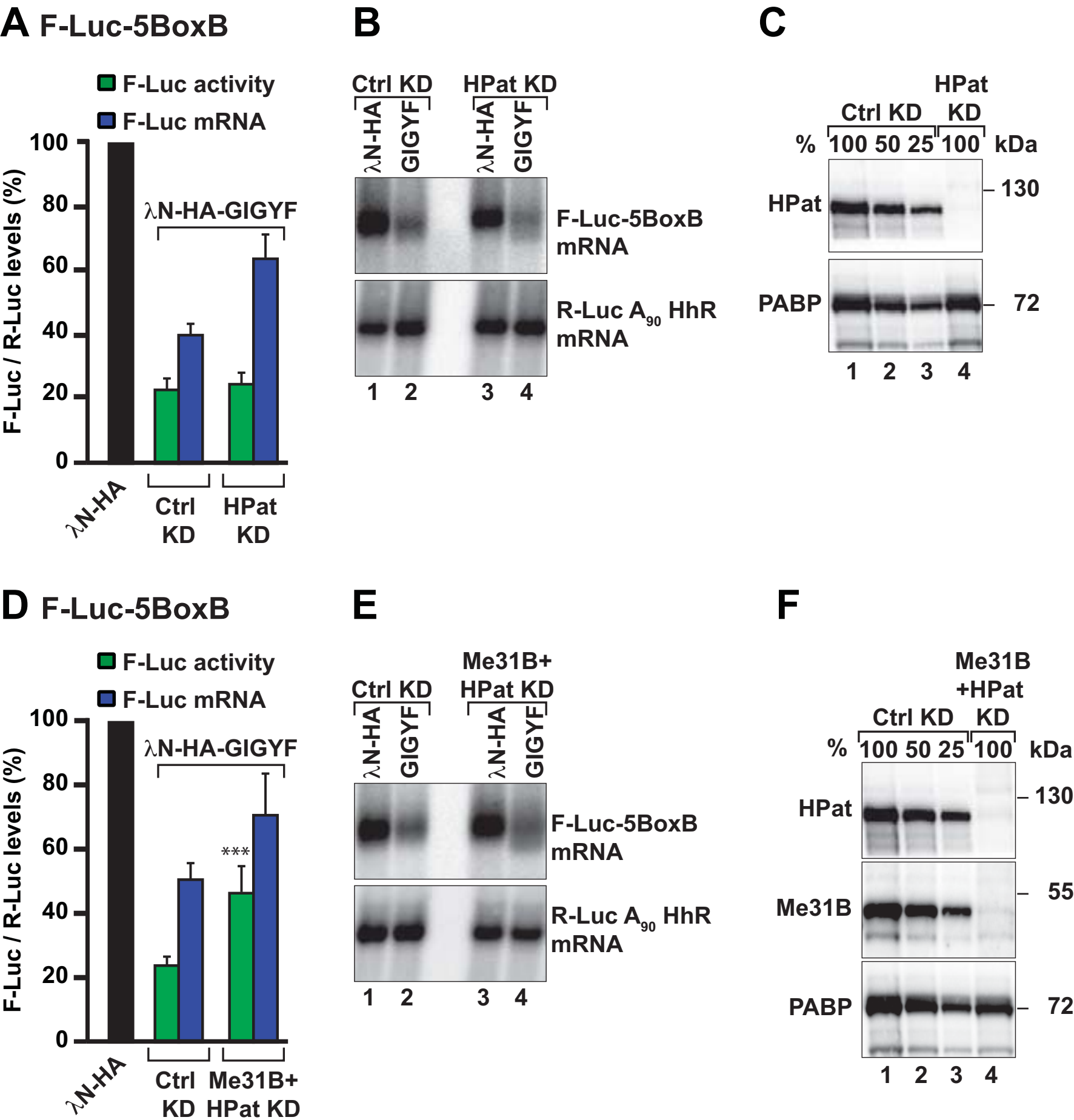

Ruscica *et al.* Fig. S10