Expanded View Figures

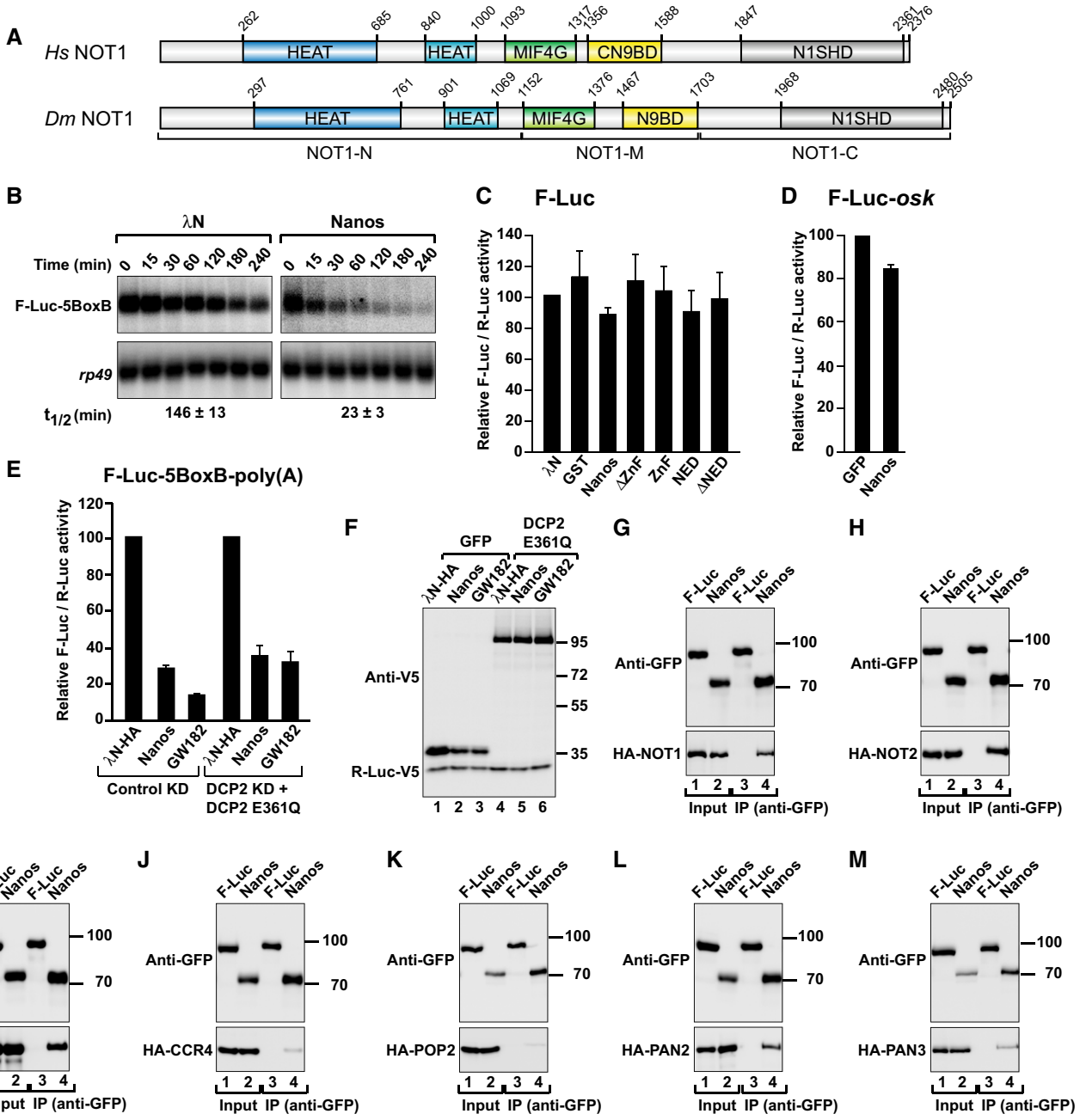

Figure EV1.

◀ **Figure EV1. *Dm* Nanos interacts with subunits of the deadenylase complexes.**

A Domain organization of *Hs* and *Dm* NOT1. NOT1 consists of N-terminal (NOT1-N), middle (NOT1-M), and C-terminal regions (NOT1-C). NOT1-N contains two HEAT repeat domains (dark and light blue); NOT1-M contains a MIF4G domain that also consists of HEAT repeats and a three-helix bundle domain (CN9BD). NOT1-C contains another HEAT repeat domain, the NOT1 superfamily homology domain (SHD).

B Northern blot analysis showing the decay of the F-Luc-5BoxB mRNA in S2 cells expressing the indicated proteins. The mRNA half-lives ($t_{1/2}$) ± standard deviations calculated from the decay curves of three independent experiments are indicated below the panels.

C Tethering assay corresponding to the experiment described in Fig 1B but using an F-Luc reporter that lacks the BoxB hairpins. F-Luc activity was normalized to R-Luc and set to 100 in cells expressing λN-HA. The panel shows mean values ± standard deviations from three independent experiments.

D GFP-tagged Nanos was coexpressed with an F-Luc reporter containing the *oskar* 3′ UTR. R-Luc served as a transfection control. F-Luc activity was normalized to that of the R-Luc transfection control and set to 100 in cells expressing GFP. The panel shows mean values ± standard deviations from three independent experiments.

E Normalized luciferase activities corresponding to the experiment shown in Fig 3A and B.

F Western blot analysis showing the expression of the DCP2 mutant (DCP2 E361Q) in the experiment described in Fig 3A and B. R-Luc-V5 served as a transfection control.

G–M Western blot analysis showing the interaction of GFP-tagged *Dm* Nanos (full length) with HA-tagged deadenylase subunits. GFP-tagged firefly luciferase (F-Luc) served as a negative control. Proteins were immunoprecipitated using a polyclonal anti-GFP antibody. Inputs and immunoprecipitates were analyzed by Western blotting using anti-GFP and anti-HA antibodies. For the GFP-tagged proteins, 3% of the inputs and 10% of the immunoprecipitates were loaded, whereas for the HA-tagged proteins, 1% of the input and 30% of the immunoprecipitates were analyzed. In each panel, cell lysates were treated with RNase A prior to immunoprecipitation.

Source data are available online for this figure.

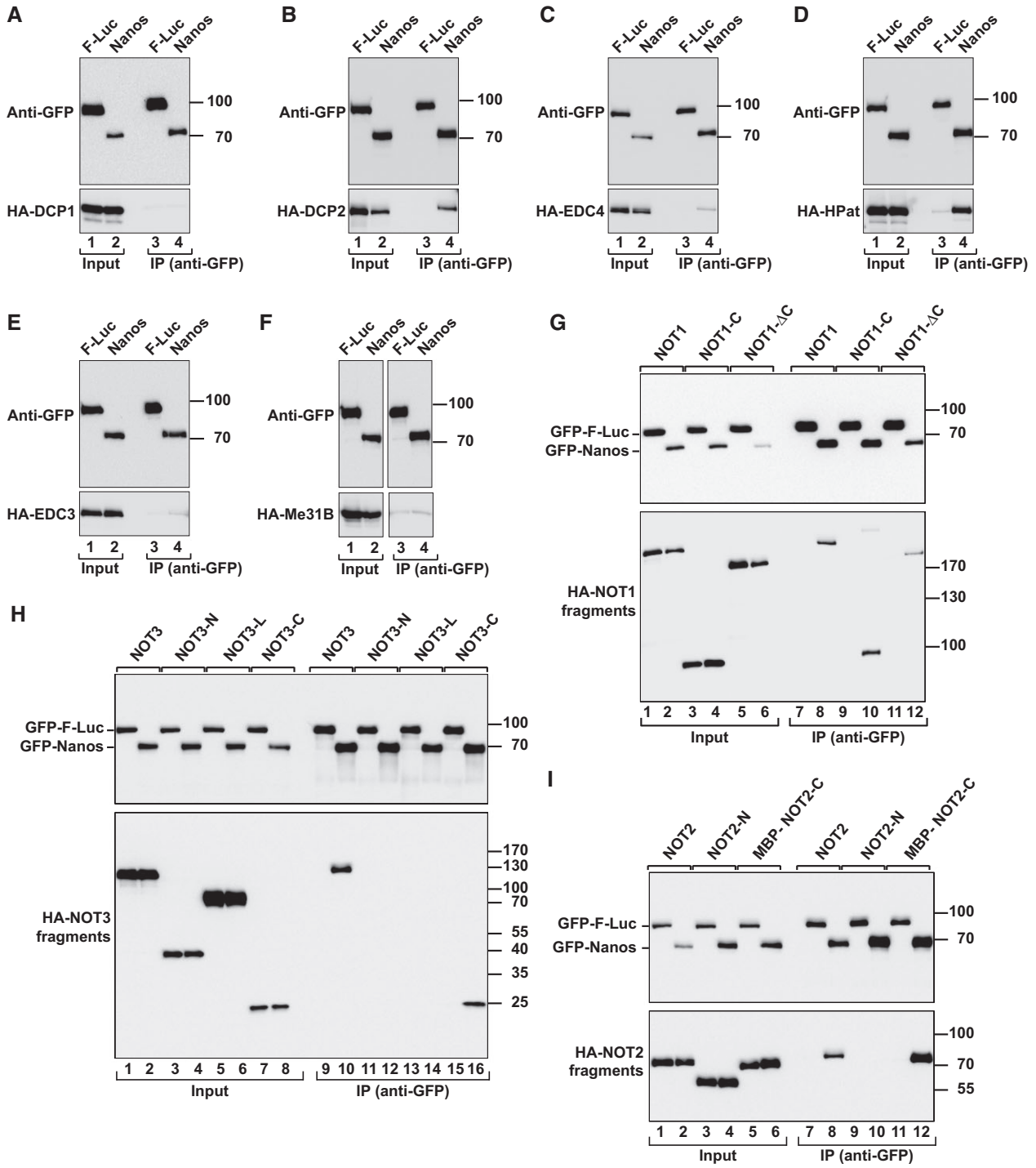

Figure EV2. *Dm* Nanos interacts with decapping factors and the NOT module.

A–F Co-immunoprecipitation assays using GFP-tagged *Dm* Nanos (full length) and HA-tagged decapping factors. Samples were analyzed as described in Fig EV1G–M.

G–I Western blot analysis showing the interaction of GFP-tagged *Dm* Nanos and HA-tagged NOT1, NOT2, and NOT3 (either full length or the indicated fragments). Proteins were immunoprecipitated from RNase A-treated cell lysates using anti-GFP antibodies. GFP-F-Luc served as a negative control. For the detection of GFP-tagged proteins, 3% of the input and 10% of the bound fractions were analyzed by Western blotting. For the detection of HA-tagged NOT1, 1.5% of the input and 35% of the bound fractions were analyzed, whereas for HA–NOT2 and HA–NOT3 proteins, 1% of the input and 30% of the immunoprecipitates were analyzed.

Source data are available online for this figure.

A Alignment *Drosophila* NED

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D.melanogaster   50 ITPVTPDPSTSAQSTHFPFLADS----------AATANSLLMQRQYHYHLLL-QQQQQLAMAQHQLALAA 108
D.anannassae     61 LTPLTPEP-TAQTTTHFPLLADS----------AAAANTLLLQRQYHYHLLLHQQQQQLALAQHQLALAA 119
D.erecta         72 LTPLTPDPSTAAQTTHFPFLADS----------AAAANTLLLQRQYHYHLLLQQQQQQLALAQHQLALAA 131
D.grimshawi      70 LTPEHS----TMSSTQFPLLTDNVQLN--------TANALLLQRQYQYHLLIQHHQQQIALAQHQLALAA 127
D.mojavensis     53 LTPE-P----TMSSTQFPLLTDSNQVNMN----ASAASTLFLQRQYQYHLLLQQQQQQLALAKHQLALAA 113
D.sechellia      72 ITPVTPDPSTSAQSTHFSFLADNN----------AATANSLLLQRQYHYHLLL-QQQQQLAMAQHQLALAA 131
D.simulans       53 ITPVTPDPSTSAQSTHFSFLADNN----------AATANSILLQRQYHYHLLL-QQQQQLAMAQHQLALAA 112
D.virilis        53 LTPE-P----TMSSTQFPLLTDSNHVN--MNA-STAASTLLMQRQYQYHLLLQQQQQQLAFAQHQLALAA 114
D.willistoni     68 LTPESPAPFGSQAATQFPLLTDNANVN-AMNA-ATAANTLLFQRQYHYHLLIQQQQQQLALAQHQLALAA 135
D.yakuba         72 LTPLTPDPTTAAQTTHFPFLADS----------AAAANTLLLQRQYHYHLLLQQQQQQLALAQHQLALAA 131
D.pseudoobscura  63 -TPLTPEPPQYGSQTQFPLLTGNSNMNMNMNMGLHTGQNMLLHQHYQYHLIIQQQQQQLALANHHLALAA 131
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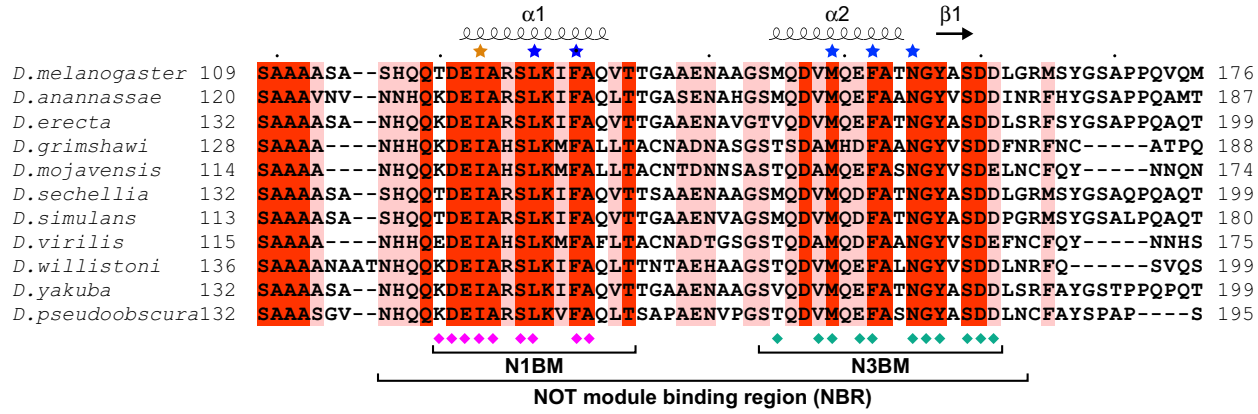


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D.melanogaster  109 SAAAASA--SHQQTDEIARSLKIFAQVTTGAAENAAGSMQDVMQEFATNGYASDDLGRMSYGSAPPQVQM 176
D.anannassae    120 SAAAVNV--NNHQKDEIARSLKIFAQLTTGASENAHGSMQDVMQEFAANGYVSDDINRFHYGSAPPQAMT 187
D.erecta        132 SAAAASA--NHQQKDEIARSLKIFAQVTTGAAENAVGTVQDVMQEFATNGYASDDLSRFSYGSAPPQAQT 199
D.grimshawi     128 SAAAA----NHHQKDEIAHSLKMFALLTACNADNASGSTSDAMHDFAANGYVSDDFNRFNC-----ATPQ 188
D.mojavensis    114 SAAAA----NHQQKDEIAHSLKMFALLTACNTDNNSASTQDAMQEFASNGYVSDELNCFQY-----NNQN 174
D.sechellia     132 SAAAASA--SHQQTDEIARSLKIFAQVTTSAAENAAGSMQDVMQDFATNGYASDDLGRMSYGSAQPQAQT 199
D.simulans      113 SAAAASA--SHQQTDEIARSLKIFAQVTTGAAENVAGSMQDVMQDFATNGYASDDPGRMSYGSALPQAQT 180
D.virilis       115 SAAAA----NHHQEDEIAHSLKMFAFLTACNADTGSGSTQDAMQDFAANGYVSDEFNCFQY-----NNHS 175
D.willistoni    136 SAAAANAATNHQQKDEIARSLKIFAQLTTNTAEHAAGSTQDVMQEFALNGYVSDDLNRFQ------SVQS 199
D.yakuba        132 SAAAASA--NHQQKDEIARSLKIFAQVTTGAAENAAGSVQDVMQEFATNGYASDDLSRFAYGSTPPQPQT 199
D.pseudoobscura 132 SAAASGV--NHQQKDEIARSLKVFAQLTSAPAENVPGSTQDVMQEFASNGYASDDLNCFAYSPAP----S 195
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D.melanogaster  177 PPQQQHQQQQGLHLPLGRNPAQLQTNGGNLMPI----PLATHWLN--NYREHLNNVWR--NMSYIPAA  236
D.anannassae    188 PPPP---PPPSLQAPLGHTSSPQASPNVNLMPVSVPAPLTAHWLS--NYREQLNNVWR--HMSYMPTA  248
D.erecta        200 PPPSQQQQQQQGLHLPLGRNTAQLHSNGANLMSN----PLATHWLN--NYREQLNNVWR--NMSYMPAV  259
D.grimshawi     189 PQQPQQQLLRQQFPSATTTVAGNSANAAGVLPS---PLAATQ-LNLNNW-EHLNNVWRSMSMAYMPNN  251
D.mojavensis    175 TVTPQSSPLPRQYP--AGNSTASATAAVSFVPP---SLAAAHWLNFNNYREHLNNVWR--SMSYVPNL  235
D.sechellia     200 QPQQQHQQQQGLHLPLGRNPAQLQVNGGNLMPI----PLATHWLN--NYREHLNNVWR--NMSYVPAA  259
D.simulans      181 QPQQQHQQQQGLHLPLGRNPAQLQVNGGNLMPM----PLATHWLN--NYREHLNNVWR--NMSYVPAA  240
D.virilis       176 SVAPQSSQLPSQVPISSAGTGGNATAEGATFPS---SLAAAHWINLSNYREHLNHVWRTMSMSYMPNV  240
D.willistoni    200 PQLQQRHLTPTFTAASGSAGANMAAATANAFPM-----TTANWLY--NYREQLNSVWR--NMSYMPAA  258
D.yakuba        200 PPPS--QQQQGMHLPLGRSPVQLQSNGANLMSN----PLATHWLN--NYREQLNNVWR--NMSYMPAA  257
D.pseudoobscura 196 PANQLHRNLPAAGAAANHPP--------QVVPA--PAPLSARWLQ--NYREQLNNVWR--SMAYMPCI  249
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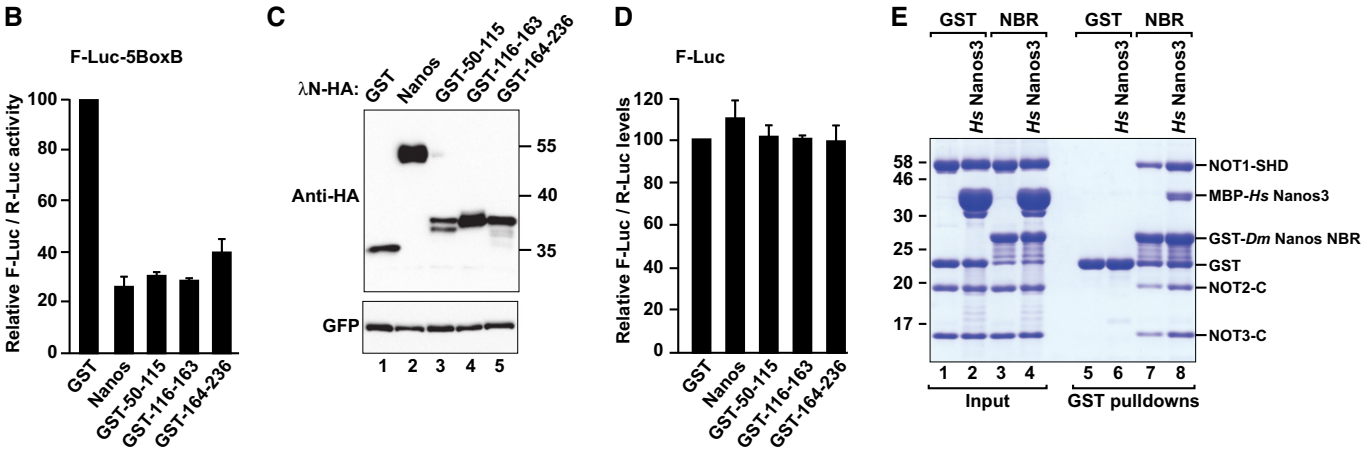

Figure EV3.

Figure EV3. Sequence alignment of the Nanos NED regions of the indicated *Drosophila* species.

A The residues conserved in all of the aligned sequences are shown with a red background, and the residues with > 70% similarity are highlighted with a salmon background. The residues interacting with NOT1 and NOT3 are indicated by magenta and cyan diamonds, respectively, including main-chain and minor side-chain contacts. The residues mutated in this study are indicated by asterisks colored in blue (mutations that disrupt NOT module binding) and in orange (I123M mutation for the incorporation of selenomethionine as an anomalous scatterer).

B Tethering assay using the F-Luc-5BoxB reporter in S2 cells expressing the indicated λN-HA-tagged NED fragments. A plasmid-expressing R-Luc mRNA was used as a transfection control. The F-Luc activities were normalized to those of the R-Luc transfection control and set to 100 in the presence of the λN-HA–GST. The panel shows mean values ± standard deviations from three independent experiments.

C Western blot analysis showing the expression of the λN-HA-tagged proteins used in the experiment described in (B). GFP served as a transfection control.

D An experiment similar to that described in (B) was performed using an F-Luc reporter that lacks the BoxB hairpins. The panel shows mean values ± standard deviations from three independent experiments.

E GST pull-down assay showing that the MBP-tagged Nanos3 NIM peptide does not compete with the GST-tagged Nanos NBR for binding to the purified NOT module.

Source data are available online for this figure.

Figure EV4. Comparison of NOT module conformations and details of *Dm* Nanos NBR binding.

A Superposition of the mutant NOT module (apo form) crystallized in space group $P2_1$ (blue; chains A, B, C), and the wild-type NOT module crystallized in space group $P2_12_12$ (red; PDB code 4C0D; Boland *et al*, 2013).

B Superposition of the mutant NOT module structure obtained in the absence (blue, chains A, B, C) and presence of the *Dm* Nanos NBR (orange; chains A, B, C). The NBR peptide is shown in red.

C Superposition of the two NOT module complexes from the asymmetric unit of the crystals obtained in the presence of the *Dm* Nanos NBR peptide. Complex 1 (chains A, B, C) is colored in orange and complex 2 (chains E, F, G) in green. The NBR peptide is shown in red and dark green.

D Crystal packing of the NOT module mutant bound to the *Dm* Nanos NBR peptide.

E Orientation of NOT1 helix α23 in the wild type (PDB code 4C0D; Boland *et al*, 2013) and mutant NOT module apo complexes. Colors are as in (A). The black lines indicate the change in the relative orientation of the helix axes.

F Orientation of the NOT1 helix α23 in the mutant NOT module complex as compared to the orientation in the complex of the NOT1 SHD with the human Nanos1 NIM (PDB code 4CQO; Bhandari *et al*, 2014).

G Alternative view of the N1BM binding pocket centered on *Dm* Nanos F130. Selected residues of NOT1 and of the NBR peptide are shown as gray and red sticks, respectively. Residues mutated in this study are underlined.

H Alternative view of the N3BM binding pocket centered on *Dm* Nanos F152. Selected residues of NOT3 and of the NBR peptide are shown as cyan and red sticks, respectively. Residues mutated in this study are underlined.

I Additional close-up of the N3BM binding site emphasizing the role of NOT3 K737 with hydrogen bonds as dashed green lines. Residues mutated in this study are underlined.

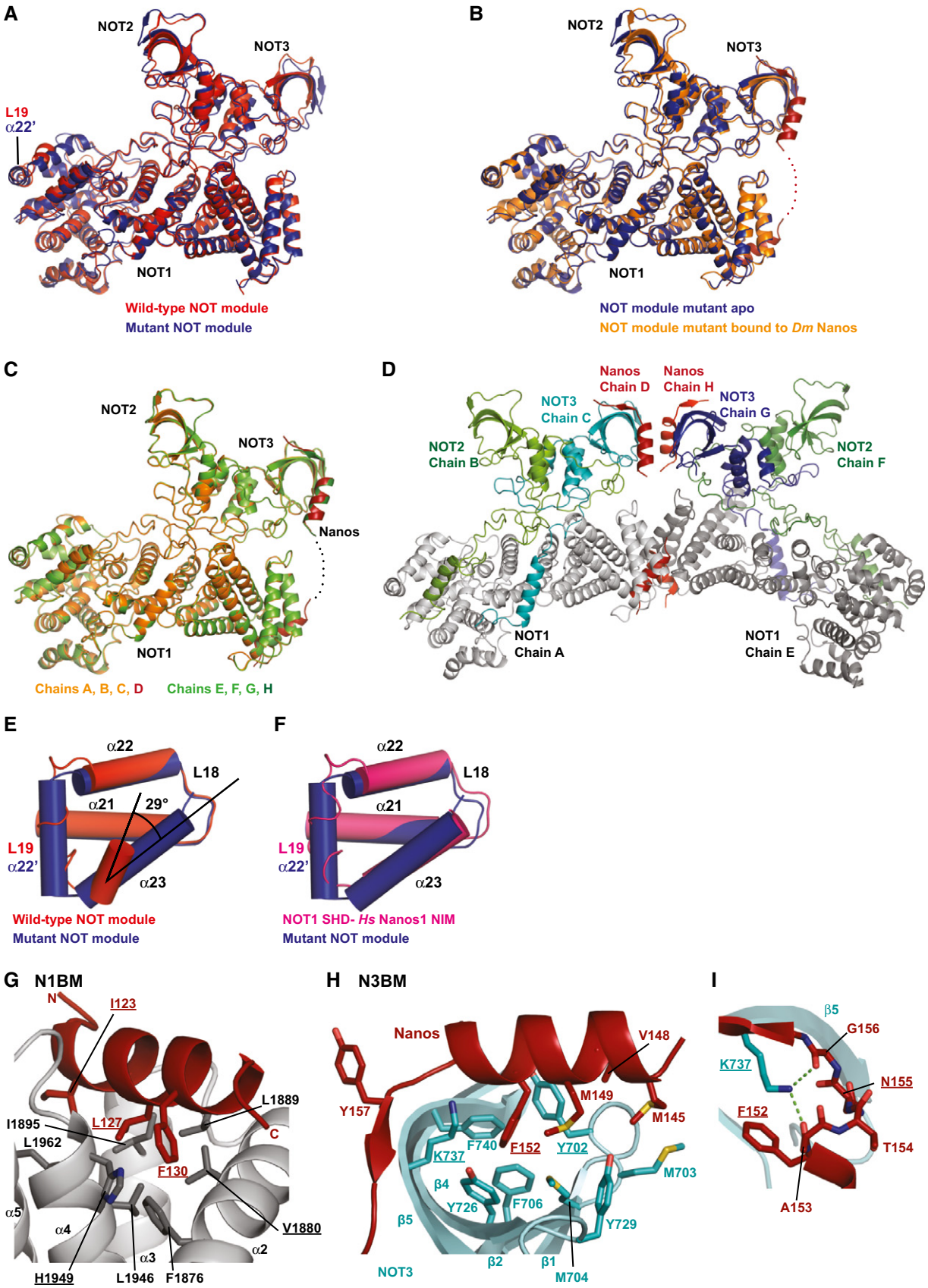

Figure EV4.

Figure EV5. Validation of the sequence assignment of the NBR peptide bound to the NOT module and activity of NBR mutants.

A–D Anomalous difference Fourier map (black mesh) calculated at a resolution of 7.5 Å and contoured at the 4.0 σ level. Data (available as source data) were collected at the Selenium K-edge peak wavelength from a crystal containing selenomethionine-substituted *Dm* Nanos NBR peptide (I123M mutant). The panels show close-up views of the binding sites of the N1BM (A, B) and the N3BM (C, D) in the same orientations as in Fig 6. Residues mutated in this study are underlined.

E–G Western blot analysis showing the interaction of GFP-tagged *Dm* Nanos (wild type or 3xMut) with HA-tagged NOT1, NOT2, and NOT3. GFP-tagged firefly luciferase (F-Luc) served as a negative control. Proteins were immunoprecipitated using a polyclonal anti-GFP antibody. Inputs and immunoprecipitates were analyzed by Western blotting as described in Fig EV1G–M.

H A tethering assay using the F-Luc-5BoxB reporter and the indicated λN-HA-tagged proteins was performed in S2 as described in Fig 1B. The panel shows mean values ± standard deviations from three independent experiments.

I Tethering assays in human HEK293T cells, using a β-globin reporter containing 6 binding sites (6xbs) for the MS2 protein and MS2-HA-tagged *Hs* Nanos2 (wild type or the indicated variants, Nanos2 ΔNIM and Nanos2 NIM-to-NBR). In the Nanos2 NIM-to-NBR protein, the NIM was replaced by the *Dm* Nanos NBR. A plasmid expressing an mRNA lacking MS2 binding sites (Control) served as a transfection control. The β-globin-6xbs mRNA levels were normalized to those of the control mRNA and set to 100 in the presence of MS2-HA. The panel shows mean values ± standard deviations from three independent experiments.

J Northern blot of representative RNA samples corresponding to the experiment shown in (I).

K Western blot analysis showing the expression of the MS2-tagged proteins used in the experiment shown in (I) and (J). V5-MBP served as a transfection control.

L Co-immunoprecipitation assay in human HEK293T cells showing the interaction of V5-SBP-tagged Nanos2 (wild type or the indicated variants) with endogenous NOT1 and NOT3. V5-SBP-tagged GFP-MBP served as a negative control.

Source data are available online for this figure.

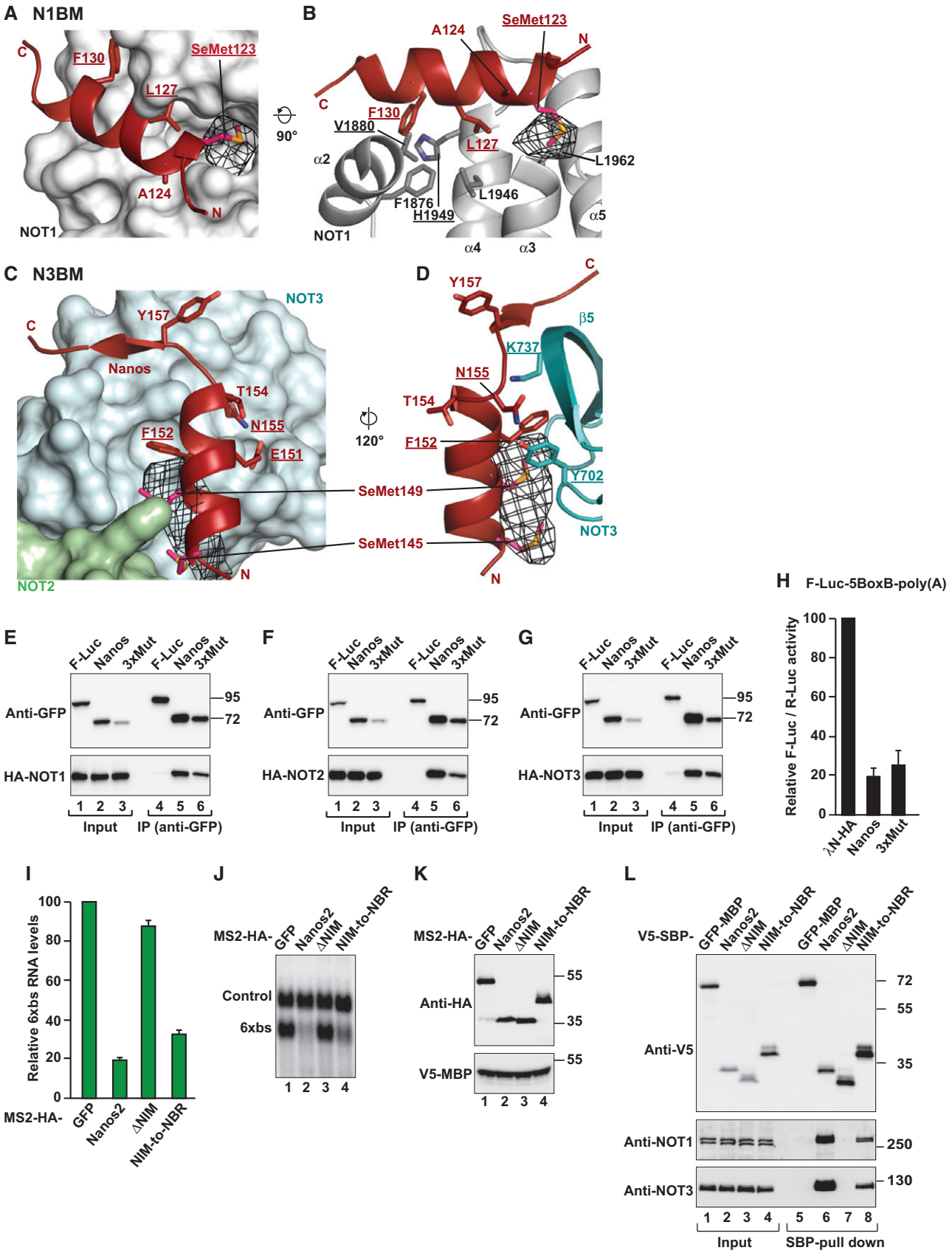

Figure EV5.