Appendix

Distinct modes of recruitment of the CCR4-NOT complex by *Drosophila* and vertebrate Nanos

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Appendix Supplementary Materials and Methods

DNA constructs

Luciferase reporters and plasmids for the expression of GFP- or HA-tagged subunits of the CCR4-NOT and PAN2-PAN3 deadenylase complexes, decapping factors, DCP2 catalytic mutant (E361Q) and GW182 were previously described (Behm-Ansmant *et al*, 2006; Tritschler *et al*, 2008; Haas *et al*, 2010). An F-luc-*hb* reporter was generated by inserting the *hunchback* 3' UTR (CG9786) into the NheI and XhoI restriction sites of plasmid pAc5.1-F-Luc (Behm-Ansmant *et al*, 2006). Plasmids for the expression of GFP and λ N-HA tagged *Dm* Nanos (Uniprot A0A0B4KGY5-1) were obtained by inserting the cDNA corresponding to the Nanos fragments were amplified by PCR using the full-length Nanos template and inserted into the same vectors. Deletion constructs were made by site-directed mutagenesis using appropriate primers. To generate the chimeric NIM-ZnF construct, we inserted a cDNA corresponding to the *Hs* Nanos2 NIM motif (codon-optimized for expression in *Dm*) followed by a Gly-Ser-Ser-Gly linker between the GST and *Dm* Nanos ZnF sequences of the pAC5.1- λ NHA-GST-ZnF plasmid.

For the expression of recombinant proteins in *E. coli*, synthetic cDNAs (codon-optimized for expression in *E. coli*) corresponding to *Dm* Nanos fragments were inserted into the XhoI and BamHI restriction sites of the pnEA-pG plasmid (Diebold *et al*, 2011), generating protein fusions containing N-terminal GST tags cleavable by the HRV3C protease. The NED and NED- Δ NBR constructs contain a C-terminal GB1 tag (Chen and Patel, 2004) fused to the Nanos sequences by a Gly-Ser-Ser-Gly linker.

Plasmids for the expression of the *Hs* NOT1 SHD and the NOT2 and NOT3 C-terminal regions have been previously described (Boland *et al*, 2013; Bhandari *et al*, 2014). Human NOT2 and NOT3 were expressed from a bicistronic plasmid based on the pnEA vector

(Diebold *et al*, 2011) and contained HRV3C-cleavable MBP and 6xHis tags, respectively. The DNA constructs used in this study are listed in Appendix Table S1.

mRNA half-live

For the measurement of mRNA half-lives, transfected cells were treated with actinomycin D (5µg/ml final concentration) 3 days after transfection, and harvested at the time points indicated. RNA samples were analyzed by Northern blot. mRNA reporter levels were normalized to the levels of rp49 mRNA and were plotted against time. The mRNA half-lives $(t_{1/2}) \pm$ standard deviations were calculated from the decay curves (not shown) obtained from three independent experiments and are indicated below the panels.

Protein expression and purification

All proteins for crystallization and *in vitro* pulldown assays were expressed in *E. coli* BL21 (DE3) Star cells (Invitrogen) in ZY medium at 20 °C overnight. *Dm* Nanos constructs were expressed with N-terminal GST tags. The NED constructs carried, in addition a C-terminal noncleavable GB1 tag. The cells were resuspended and lysed in binding buffer containing 50 mM HEPES (pH 7.5), 300 mM NaCl and 2 mM dithiothreitol (DTT) supplemented with protease inhibitors, lysozyme and DNaseI. The proteins were isolated from the crude lysate using Protino glutathione agarose 4B beads (Macherey Nagel) and eluted in binding buffer containing 50 mM glutathione. For GST pulldown assays, the proteins were further purified by anion exchange chromatography using a HiTrap Q column (GE Healthcare) followed by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) in a buffer containing 10 mM HEPES (pH 7.5), 200 mM NaCl and 2 mM DTT. For crystallization, the GST tag was cleaved after elution from the glutathione beads by incubating overnight with

recombinant HRV3C protease. The protein was separated from the tag by gel filtration on a Superdex 75 26/60 column (GE Healthcare).

The assembled *Hs* NOT module was obtained by co-expression of MBP-tagged NOT1 SHD (residues 1833–2361), MBP-tagged NOT2 (residues 350–540) and His₆-tagged NOT3 (residues 607–748). The cells were lysed in lysis buffer supplemented with DNaseI, lysozyme and protease inhibitors. The protein complex was purified over amylose resin and eluted with lysis buffer supplemented with 25 mM D-(+)-maltose and 20 mM imidazole. The NOT module was further purified via nickel affinity chromatography using a HiTrap IMAC column (GE Healthcare). The affinity tags were removed by overnight cleavage using HRV3C protease during dialysis in a buffer containing 50 mM Tris-HCl (pH 8.6), 200 mM NaCl, 10% glycerol and 2 mM DTT. The cleaved MBP tags were removed by binding to amylose resin. The remaining contaminants were removed by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) in a buffer containing 10 mM Tris-HCl (pH 8.6), 200 mM NaCl, 10% glycerol and 2 mM DTT.

Crystallization

Initial screens were carried out using the sitting drop vapor diffusion method using 5 mg/ml of the *Hs* NOT module or a mixture containing the *Hs* NOT module (5 mg/ml) and a 1.5-fold molar excess of the *Dm* Nanos NBR peptide. Samples (200 nl) were preincubated for 1 hr in a buffer containing 10 mM Tris-HCl (pH 8.6), 200 mM NaCl, 10% glycerol and 2 mM DTT and were added to 200 nl of reservoir solution. Crystals appeared within three days in many different conditions containing polyethylene glycol (PEG). The best NOT module crystals were optimized to grow in 0.2 M sodium acetate, 0.1 M sodium citrate (pH 5.5) and 10% (w/v) PEG 4000. The best-diffracting crystals of the NOT module bound to the Nanos peptide were optimized to grow over a week in 100 mM MES (pH 6.0), 260 mM LiCl and 18.6% (w/v) PEG

6000. Crystals were cryoprotected using reservoir solution supplemented with 15% glycerol and flash-frozen in liquid nitrogen.

Data collection and structure determination

Diffraction data were recorded on a PILATUS 6M detector at the PXII beamline of the Swiss Light Source (SLS) at a temperature of 100 K. Data were processed using XDS and XSCALE (Kabsch, 2010). Initial phase information was obtained by molecular replacement with the structure of the *Hs* NOT module (PDB code 4C0D) as a search model using PHASER (McCoy *et al*, 2007) from the CCP4 package (Winn *et al*, 2011). The models were then improved by iterative cycles of refinement using PHENIX (Afonine *et al*, 2012) and BUSTER (Bricogne *et al*, 2011) and manual building in COOT (Emsley *et al*, 2010). Finally, the *Dm* Nanos NBR was built into the density and improved by several additional refinement cycles. Reported coordinate errors (Table 1) are from BUSTER and correspond to the diffraction-component precision index (Blow, 2002).

Anomalous difference Fourier map

Anomalous data were also recorded at the PXII beamline, at a wavelength of 0.979 Å and to a resolution of 3.9 Å. Data were processed and scaled using XDS and XSCALE, keeping Friedel mates apart to extract the anomalous difference for the calculation of the map coefficients. Phases were obtained from molecular replacement (PHASER) using the refined structure of the complex and searching for two copies, followed by one cycle of rigid body refinement in PHENIX.

Sequence searches and alignments

Nanos and NOT1-3 protein sequences retrieved from TREEFAM were (http://www.treefam.org) and aligned using the MAFFT webserver (http://mafft.cbrc.jp; L-INS-i preset) from within JALVIEW (http://www.jalview.org). Positional conservation and SCORECONS similarity calculated using the webserver scores were (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl) with default settings. Alignments were illustrated manually.

Appendix References

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Appendix Figure S1. Raisch et al.

NOT1		
	$\alpha 1$ L1 $\alpha 2$ L2 $\alpha 3$	
HS Dm	1840 SEYDDPPGLREKAEYLLREWVNLYH-SAAAGRDSTKAFSAFVGQMHQQGILKTDDLITRFFRLCTEMCVEI 1961 CDTDDPPGLOEKTEFLLKDWVALYTOONOOSTRDARNFGAFVOKMNTYGILKTDDLITRFFROATHICTDV	VYRMFA 2037
Ag	1665 SDIDDSPGFLERAEFLLKDWVTIAL-SPNTCRDPLKGFSVFVGKMNAHGILKGDEPLTRFFRFATQYCIDL	TYRNMN 1740
BM	$1831 \ \texttt{QNYDD} \texttt{PPGLQEKTENLLREWRNVLL-SPLTEIELAQNFNLYVHRMNNnGILKSDDMIARFFRMASQMCIEN}$	VY <mark>Q</mark> LLN 1906
Dr	1840 SEYDDPPGLREKAEYLLREWVNLYH-SAAAGRDSTKAFSAFVGQMHQQGILKTDDLITRFFRLCTEMCVEI	SYRAQA 1915
Ce	1965 RGYDDQ-EMTAKVEIIMREWIGLCY-SPTGQRSPQESLAQMIQLMHEHGVLATDDKITQFFRLCVENCVDI	SVRVMK 2039
	α3 L3 α4 L4 α5 L5	α6
HS	$1916 \ {\tt eqqhnpaanptmirakcyhnldafvrliallvkhsgeatntvtkinllnkvlgivvgvllqdhdvrqsefq}$	QLPYHR 1992
Dm	2038 EPSLPINQAKNKIFQWIDAFVHLIAMLVRHSGEAGNPTTKINLLNKVLGIVLGTLIKDHEMRGVSFQ	2VGYHR 2110
Bm	1907 EDRVNPP-PVPOKREKFYTMCDSFIKLVSLLIKNTADSGNPTPKLNLLNKILGIIVGWLLODHDOGTNFO	DLPYHR 1982
Dr	$1916 \ \mathbf{EQQH} - \mathbf{PTTSPAIIRAKCYHNL} \mathbf{DAFVRLIALLVKHSGEATNTVTKINLLNKVLGIVVGVLIQDHDVRQTEFQ}$	QLP <mark>YHR</mark> 1991
Ce	2040 SEQLANGLPTTLIRHRCYYTLDAFVKLMALMIRHS-DNGQSQNKINLLKKLLNIIVGVLHMDHEVRKQDFN	AMP <mark>YHR</mark> 2115
	~6 6 ~7 7 ~8 ~0 8	~10
		el ee
HS	1993 IFIMLLLELNAPEHVLETINFQTLTAFCNTFHILRPTKAPGFVYAWLELISHRIFIARMLAHT	PQQKGW 2061
Dm	2111 FFMMLFMELCTADVILESLMHSIVSAFAYTYHLLNPSVAPGFCFAWLELISHRVFLGRILVQI	PGQ <mark>KG</mark> W 2179
Ag	1809 IFAMLFLELTTHDPILENISISVITAFCHTFHILRPSAAPGFCYSWLELIAHRVFIGRVLAQI	PQQKGW 1877
BM Dr	1983 LLLILFLUMNMAEPVLESMNIQVLTAFCHTLRIIKFSVAPGFCIAWLEIVAHRAFINRVLAVT 1002 TETMLILEINAPEHVLETINFOTITAFCNTEHILPTKAPGFVVAWLEITSUBTFTABMLAUT	POOKGW 2051
Ce	2116 ILISLFNEITGPDPLKLLEPIAWSILEAFGQTFFALQPRRMPGFAFAWLDIVGHRNVIGRLLANTGIAETV.	DAVKTA 2192
	α10 L9 α11 α12 α13 L10 α14	L11
TT		
HS Dm	2002 PMIAQLLIDLFKILAPFLRNVELTKPMQILIKGTLRVLLVLLHDFPEFLCDYHIGFCDVIPPNCIQLRNLL 2180 PLYAOLLODLFKYLAPFLRNTFLCKPVOLLYKGTLRVLLVLHDFPEFLCDYHIGFCDTPPNCYOMPNII	LSAFPR 2138
Aq	1878 HMYSQLLIDLFKYLAPFLRNAELAKPVQHLYKGTLRVLLVLLHDFPEFLCDYHFAFCDVIPSNCIQMRNLI	LSPYPR 1954
Bm	2052 GMYSTLLIELFKFLDPFLRNTELAPPVMTLYKGTLKVLLVLLHDFPEFLCDYHYGFCDEIPPNCIQMRNLI	LSAFPR 2128
Dr	2061 PMYAQLLIDLFKYLAPFLRNVELNKPMQILYKGTLRVLLVLLHDFPEFLCDYHYGFCDVIPPNCIQLRNLI	LSAFPR 2137
Се	2193 ATYTQLIISHLKFLAPFLRNIQLPKSIAILYKGTLRVLLVILHDFPELLCEFHYVICDTIPPNCVQLRNLI	LSAYPR 2269
	L11 α15 L12 α16 L13	α17
		. ee
HS	2139 NMRLPDFFTPNLK-VDMLSEINIAPRILTNFTGVM-PPQFKKDLDSYLKTRSPVTFLSDLRSNLQVSNEPG	NRYNLQ 2213
Dm Da	2257 NMRLPDPFTPNLK-VDMLSDSSNAPKVLSSYIMNIQPANFKKDLDSYLKARAPVTFLSELRGHLQVTSEPG	TRYNMA 2332
Ag Bm	2129 NMRLPDPFTPNLK-VDLLPEIALPPRAVINYSTIISASOFKKDLDAYLKARAPVTFLSELRGNMOVANEPG	KRYNSO 2204
Dr	2138 NMRLPDPFTPNLK-VDMLSEINIAPRILTNFTGVM-PSQFKKDLDSYLKTRSPVTFLSELRSNLQVSNEPG	NRYNIQ 2212
Ce	2270 QMRLPDPFALNFKQVDTIPEMAVEPKSNLNMATII-PDNIRIPLDEYLANRISVDFLPNLPTLLQTQNQAG	FKYN TT 2345
	α17 L14 α18 L15 α19 L16 α2	20
	<u> </u>	000000
		•
HS	$2214 \ \texttt{Linalvlyvgtq} \texttt{aiahihnkgstpsmstithsahmdifq} \texttt{nlavdldtegrylflnaianqlrypnshthyf}$	SCTMLY 2290
Hs Dm	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF	SCTMLY 2290 SCAVLH 2409
Hs Dm Ag Bm	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2205 LMNAUVLYVGTQAIAHIRSKNLGPTMSTIHSAHMDIFQNFTVDFDFEGRYLFLNAIANQLRYPNSHTHYF	SCTMLY 2290 SCAVLH 2409 SCCILY 2107
Hs Dm Ag Bm Dr	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2205 LMNAVVLYVGTQAIAYIRAKGQTPNMSTIAHSAHMDIFQNFTVDFDFEGRYLFLNAIANQLRYPNSHTHYF 2213 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF	CTMLY 2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289
Hs Dm Ag Bm Dr Ce	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2205 LMNAVVLYVGTQAIAYIRAKGQTPNMSTIAHSAHMDIFQNFTVDFDFEGRYLFLNAIANQLRYPNSHTHYF 2213 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGIRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNAHTHYF	. . SCTMLY . SCAVLH . SCCILY . SCCILY . SCCLLY . SCTMLY . SCTMLY . SCTMLY . SCTMLY . SCVFLY .
Hs Dm Ag Bm Dr Ce	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2205 LMNAVVLYVGTQAIAYIRAKGQTPNMSTIAHSAHMDIFQNFTVDFDFEGRYLFLNAIANQLRYPNSHTHYF 2213 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGIRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF	SCTMLY 2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCTMLY 2289 SCVFLY 2422
Hs Dm Ag Bm Dr Ce	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2035 LMNAVVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 213 LINALVLYVGTQAIAHIRNKGSTPSMSTITHSAHMDIFQNFTVDFDFEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGIRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF 2340 117 α21 118 α22 α22' (110) α23	SCTMLY 2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCVFLY 2422
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Hs Dm Ag Bm Dr Ce Hs	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNAVVLYVGTQAIAHIRSKNLGPTMSTIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLLFNGIANQLRYPNSHTHYF 2346 L18 α22 α22' (L19) α23 000000000000000000000000000000000000	2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCVFLY 2422
Hs Dm Ag Bm Dr Ce Hs Dm	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNAVVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LINALVLYVGTQAIAHIRSKNLGPTMSTIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 213 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTQAIAHINNKGSTPSMSTITHSAMMOIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTQAIAHINNKGSTPSMSTITHSAMMOIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTQAIAHINNKGSTPSMSTITHSAMMOIFQNLAIQLDTEGRYLLFNGIANQLRYPNAHTHYF 2347 Q11 Q22 Q22 2410 L18 Q22 Q22 Q22 2291 LFAEANTEAIQEQITRVLL	2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCVFLY 2422 2361 2361 2480
Hs Dm Ag Bm Dr Ce Hs Dm Ag	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2035 LMNAVVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2036 LINALVLYVGTQAIAHIRSKNLGPTMSTIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 213 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNAHTHYF 2346 VMNALVLYVGTRAIEHLHLRRQRISTLNIAHTSYMDIFQNLAIQLDTEGRYLFNGIANQLRYPNAHTHYF 2347 L18 a22 a22'(L19) a23 2000 0000000000 0000000000 00000000000 00000000000 00000000000 2291 LFAEANTEAIQEQITRVLLERLIVNRPHPWGLLITFIELIKNPAFKFWNHEFVHCAPEIEKLFQSVAQCCM 1 2410 LFAEANSEAIQEQITRVLLERLIVNRPHPWGLLITFIELIKNPIYKFWDHDFVHCAPEITKLFESVARSCL 1 2108 LFAEANSEAIQEQITRVLLERLIVNRPHPWGLLITF	2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCVFLY 2422 2361 2480 2178
HS Dm Ag Bm Dr Ce HS Dm Ag Bm	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2035 LMNAVVLYVGTQAIAHIRSKNLGPTMSTIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 213 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2347 WMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2348 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAIQLDTEGRYLFNGIANQLRYPNSHTHYF 2349 LFAEANTEAIQEQITRVLLERLIVNRPHPWGLLITFIELIKNPIXFWHEFVHCAPEIEKLFQSVAQCCM L	2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCVFLY 2422 2361 2480 2178 2342 2361
HS Dm Ag Bm Dr Ce HS Dm Ag Bm Dr Ce	2214 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2333 LMNALVMYVGTQAIALIRNKNFVPNTSNIAHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2031 LMNALVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2035 LMNAVVLYVGTQAIAHIRSKNLGPTMSTIIHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 213 LINALVLYVGTQAIAHIRNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFLNAIANQLRYPNSHTHYF 2346 VMNALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNAIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLJDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLJDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLJDTEGRYLFNGIANQLRYPNSHTHYF 2346 LINALVLYVGTQAIAHIHNKGSTPSMSTITHSAHMDIFQNLAVDLJDTEGRYLFNGIANQLRYPNSHT 2346 LFAEANSEAIQEQITRVLLERLIVNRPHPWGLLITFIELIKNPIYKFWDHDFVHCAPEIEKLFQSVAQCCM 2423 LFAEANSEAIQEQITRVLLERLIVNRPHPWGLLITFIELIKNPIYKFWSHEFVHCAPEIEK	2290 SCAVLH 2409 SCCILY 2107 SCCLLY 2281 SCTMLY 2289 SCVFLY 2422 2361 2480 2178 2342 2360 2493

Appendix Figure S1. Sequence alignment of the NOT1 SHD.

The secondary structural elements as determined from the *Hs* NOT1 structure are shown above the alignment. The residues conserved in all of the aligned sequences are shown on a dark magenta background, and the residues with >70% similarity are shown on a light magenta background. The residues interacting with the *Dm* NBR and the human Nanos1 NIM peptide are indicated by red and orange diamonds, respectively. The residues mutated in this study are indicated by asterisks colored in blue (mutations that disrupt NBR binding) or in green (crystallization mutations). Loop L19 as observed in the previous structure of the NOT module (PDB entry 4C0D) folds as an α -helix (α 22') in the present structures. The species abbreviations are as follows: *Hs* (*Homo sapiens*), *Dm* (*Drosophila melanogaster*), *Ag* (*Anopheles gambiae*), *Bm* (*Bombyx mori*), *Dr* (*Danio rerio*), and *Ce* (*Caenorhabditis elegans*).

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A,B The secondary structural elements as determined from the *Hs* NOT module structure are shown above the alignment. The residues conserved in all of the aligned sequences are shown with a dark green (NOT2) or cyan (NOT3) background, and the residues with >70% similarity are highlighted with a light green or cyan background. The NOT3 residues

interacting with the *Dm* NBR are indicated by red diamonds. The residues mutated in this study are indicated by blue asterisks. The species abbreviations are as described in Appendix Fig S1.

Dm Nanos, isoform B (U	Jniprot A0A0B4KGY5-1)	Comment			
Full length	λN-HA-Nanos				
C	GFP-Nanos				
ΔZnF	λ N-HA-Nanos 1–296				
	GFP-Nanos 1–296				
ZnF	λ N-HA-GST-Nanos 297–381				
	GEP Nanos 207 381				
NFD	GST-Nanos 50–236-GB1				
NLD	AN HA GST Nanos 50, 236				
	GED Nanos 50, 236				
Nanos ANED	AN HA Neper ASO 226				
Inalios-AINED	CED Names A50, 226				
NED ANDD	GFP-Nanos $\Delta 50-230$				
	GST -Nalios 30–230- Δ 110–103- GBT				
INDI	AN HA COT Names 116, 162				
NDD 2M4	Λ N-HA-GS1-Nanos 110–103	Matation discusts NOT2 his disc			
NBR 3XMut	GSI-Nanos 116–163 E151A E152A N155A	Mutation disrupts NO13 binding			
	AN HA CST Names 11(1(2	Mutation disrupts NOT2 hinding			
	Λ N-HA-GSI-Nanos II0–105 E151A E152A N155A	Mutation disrupts NO15 binding			
NBR E152E	GST Nanos 116 163 F152F	Mutation disrupts NOT3 binding			
NDK F152L	$\frac{116}{162} = 1000 \times 10000 \times 1000 \times 10000 \times 1000 \times 1000 \times 1000 \times 1000 \times 10000 \times 100000 \times 10000 \times 10000 \times 10000 \times 10000 \times 10000000 \times 100000 \times 100000000$	Mutation disrupts NOT3 binding			
NDD 2vMut	CST Nanos 116 162 L 127D E120D	Mutation disrupts NOT1 binding			
NDK 2XMut	$\Delta N HA CST Names 116 163$	Mutation disrupts NOT1 binding			
	L 127D E130D	Mutation disrupts NOTT binding			
NBR 1123M	GST-Nanos 116-163 1123M	SeMet labeling			
50_115	AN HA CST Names 50, 115				
164_236	λ N HA GST Nanos 164 226				
104 230	XN-11A-031-Nallos 104-230				
Hs Nanos2 (Uniprot P60	321) - <i>Dm</i> Nanos chimera				
NIM-ZnF	λN-HA-GST- <i>Hs</i> Nanos2 NIM- <i>Dm</i>				
	Nanos 297–381				
NIM ' ZnF	λN-HA-GST- <i>Hs</i> Nanos2 NIM				
	(F6A,W9E)- <i>Dm</i> Nanos 297–381				
Hs Nanos3 (Uniprot P60	323)				
NIM	MBP-Hs Nanos3 4–20-Strep				
Hs NOT1 (CNOT1 Unit	prot A5VKK6)				
MBP-NOT1-SHD	MBP- <i>H</i> s NOT1 1833_2361				
MBP-NOT1-SHD FFF	MBP- <i>Hs</i> NOT1 1833–2361	Crystallization mutant L19			
	H2344E.C2345E.A2346E	Crystanization indunit Ery			
MBP-NOT1-SHD	MBP- <i>Hs</i> NOT1 1833–2361 V1880E	Disrupts Nanos binding			
V1880E		1			
MBP-NOT1-SHD	MBP- <i>Hs</i> NOT1 1833–2361 H1949D	Disrupts Nanos binding			
H1949D					
· · ·					
Hs NOT2 (CNOT2, Uniprot Q9NZN8)					
NOT2-C	MBP- <i>Hs</i> NOT2 350–540				

Appendix Table S1. Constructs used in this study.

Hs NOT3 (CNOT3, Uniprot O75175)						
NOT3-C	6xHis-Hs NOT3 607–748					
NOT3-C Y702A	6xHis-Hs NOT3 607–748 Y702A	Disrupts Nanos binding				
Dm NOT1, isoform C (Uniprot A8DY81)						
NOT1-C V2002E	λN-HA-NOT1 1710–2505 V2002E	Disrupts Nanos binding				
NOT1-C H2067D	λN-HA-NOT1 1710–2505 H2067D	Disrupts Nanos binding				
NOT1-C	λΝ-ΗΑ-ΝΟΤ1 1710–2505					
NOT1- ΔC	λΝ-ΗΑ-ΝΟΤ1 Δ1710–2505					
Dm NOT3 (Uniprot Q7K	(126)					
NOT3-N	λΝ-ΗΑ-ΝΟΤ3 1–246					
NOT3-L	λΝ-ΗΑ-ΝΟΤ3 239–681					
NOT3-C	λΝ-ΗΑ-ΝΟΤ3 677–844					
Dm NOT2 (Uniprot Q94	547)					
NOT2-N	λN-HA-NOT2 1–401					
NOT2-C	λΝ-ΗΑ-ΝΟΤ2 402–585					
Dm hunchback (Flybase	ID: FBgn0001180)					
F-Luc-hb 3'UTR	F-Luc-hunchback 3'UTR					
ΔBoxA	F-Luc-hunchback-					
	3'UTR Δ(2495–2499)nt					
	$\Delta(2546-2550)$ nt					
ΔBoxB	F-Luc-hunchback-					
	3'UTR Δ(2505–2510)nt					
	$\Delta(2556-2561)$ nt					

Antibody	Source	Catalog Number	Dilution	Monoclonal/ Polyclonal
Anti-HA-HRP (for western blots)	Roche	12 013 819 001	1:5,000	Monoclonal
Anti-HA (for immunoprecipitations)	Covance	MMS-101P		Mouse monoclonal
Anti-GFP (for western blots)	Roche	11 814 460 001	1:2,000	Mouse monoclonal
Anti-GFP (for immunoprecipitations)	In house	Tritschler <i>et al</i> , 2008		Rabbit polyclonal
Anti-mouse IgG-HRP	GE Healthcare	NA931V	1:10,000	Sheep polyclonal
Anti-rabbit IgG-HRP	GE Healthcare	NA934V	1:10,000	Donkey polyclonal
Anti- <i>Dm</i> NOT1	Kind gift from E. Wahle	Jeske <i>et al</i> , 2006	1:1,000	Rabbit polyclonal
Anti- <i>Dm</i> NOT2	Kind gift from E. Wahle	Jeske <i>et al</i> , 2006	1:3,000	Rabbit polyclonal
Anti- <i>Dm</i> NOT3	Kind gift from E. Wahle	Jeske <i>et al</i> , 2006	1:3,000	Rabbit polyclonal
Anti-tubulin	Sigma Aldrich	T6199	1:10,000	Mouse monoclonal

Appendix Table S2. Antibodies used in this study.