SUPPLEMENTARY APPENDIX FOR

Hit and run vs long-term activation of PARP-1 by its different domains fine-tunes nuclear processes

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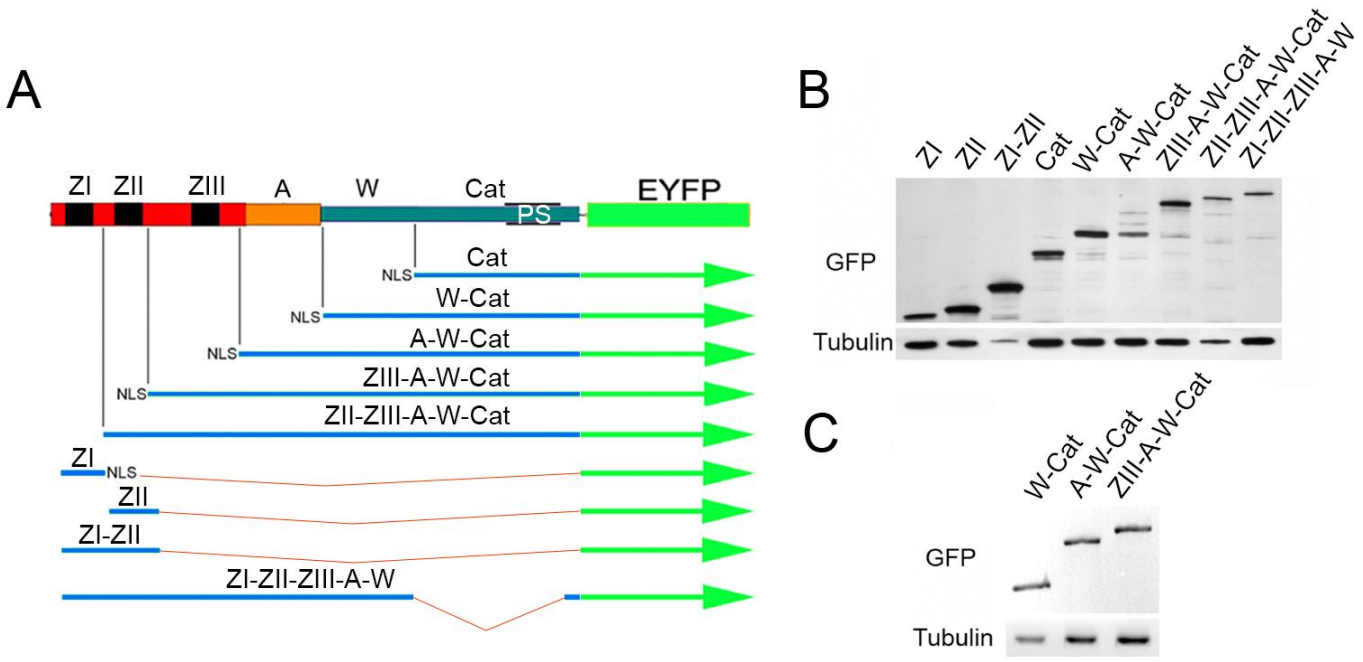
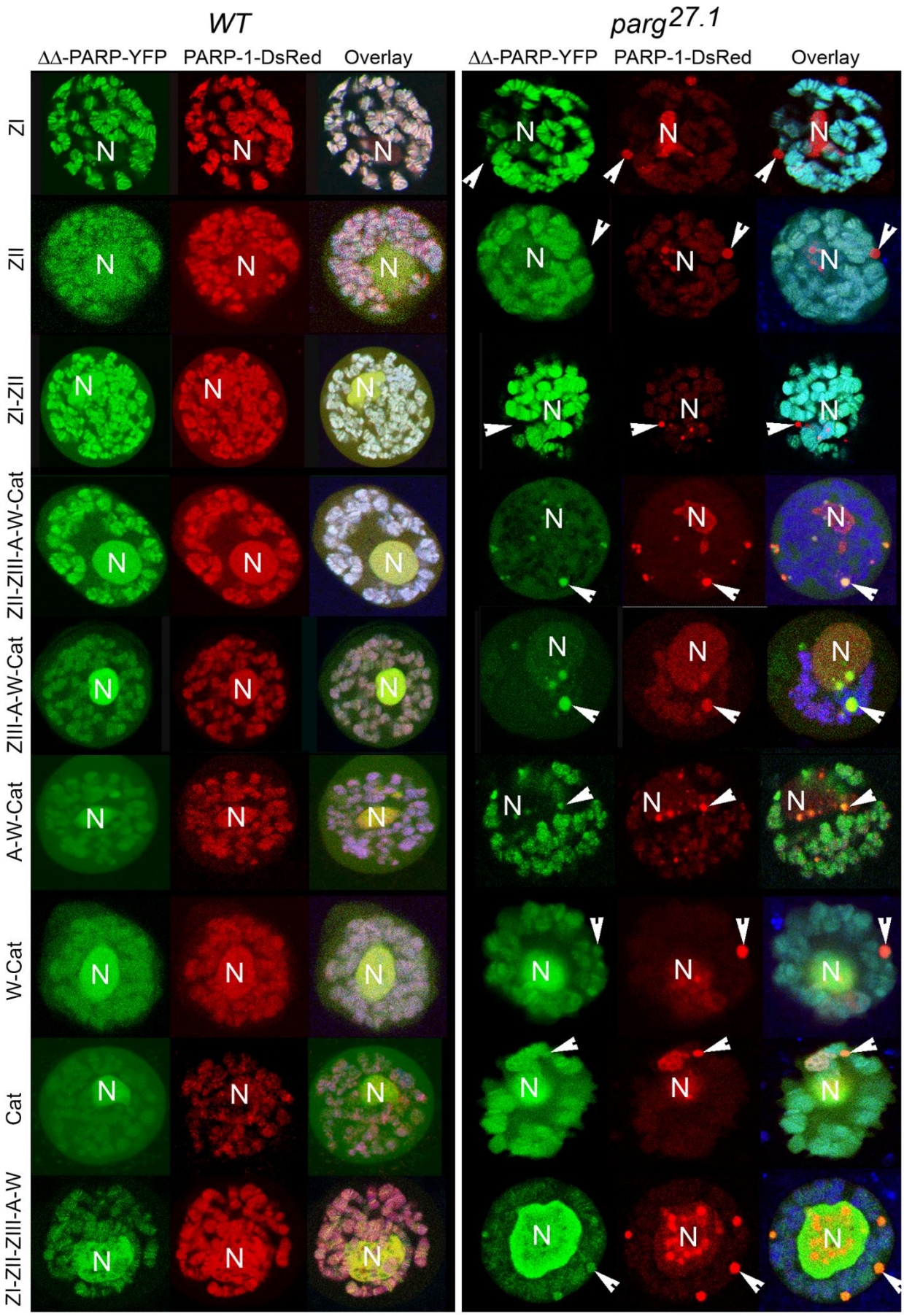
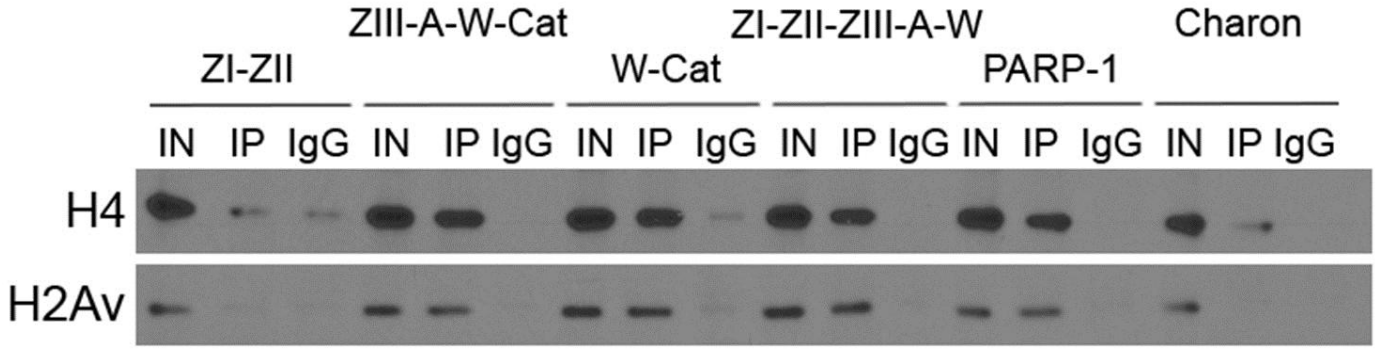


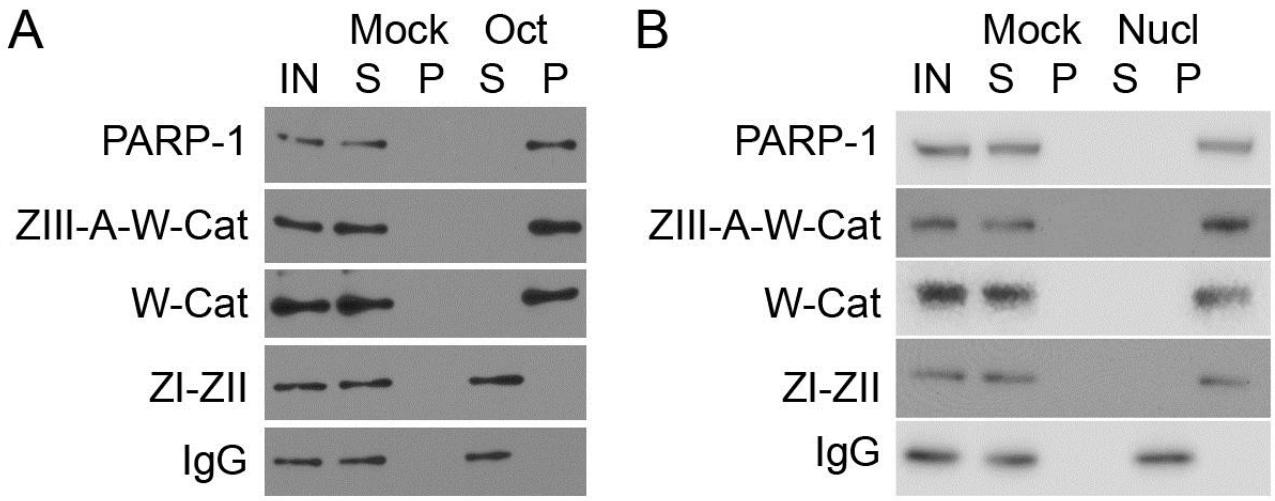
Supplemental Figure S1. Recombinant deletional PARP-1 isoforms demonstrate stability when expressed in the parg27.1; parpC03256 double mutant *Drosophila* tissues **A.** Composition of recombinant transgenic PARP-1 constructs for *in vivo* experiments. **B.** The expression of recombinant PARP-1 isoforms in *Drosophila* third instar larvae. Western blot analysis using anti-GFP antibody is presented. Anti-Tubulin antibody was used as a loading control. **C.** Although the A-W-Cat -EYFP construct demonstrates instability when expressed in whole *Drosophila* (Panel **B**), it is stable in *Drosophila* salivary glands. Western blot analysis of proteins extracted from 40 pairs of salivary glands. In all experiments described in the Results section, we used salivary gland tissues only.


WT
parg27.1
ΔΔ-PARP-YFP
PARP-1-DsRed
Overlay
ΔΔ-PARP-YFP
PARP-1-DsRed
Overlay
ZI
ZII
ZI-ZII
ZII-ZIII-A-W-Cat
ZIII-A-W-Cat
A-W-Cat
W-Cat
Cat
ZI-ZII-ZIII-A-W
N

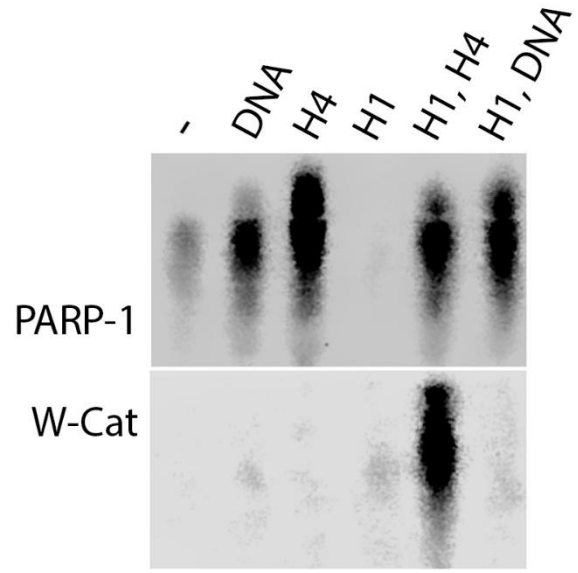
Supplemental Figure S2. *In vivo* localization of recombinant deletional PARP-1 isoforms in the wild-type *Drosophila* salivary gland polytene nuclei. Left: Live dissected larval salivary glands expressing full-length PARP-1-DsRed (Red) and PARP-1 deletional isoforms (ΔΔ-PARP-YFP) (green) were stained with the DNA binding dye Draq5 (blue, shown only in Overlay) and analyzed by confocal microscopy live imaging. A single nucleus is shown for each experiment. Positions of nucleoli are indicated with arrows. **Right:** *in vivo* localization of recombinant PARP-1 isoforms in mutant *parg*$^{27.1}$ *Drosophila* salivary gland polytene nuclei. In *parg*$^{27.1}$ nuclei, most pADPr-modified and bound proteins accumulate in Cajal bodies (1). Live dissected larval salivary glands of *parg*$^{27.1}$ mutants expressing full-length PARP-1-DsRed (Red) and PARP-1 deletional isoforms (ΔΔ-PARP-YFP) (green) were stained with the DNA binding dye Draq5 (blue, shown only in Overlay) and analyzed by confocal microscopy live imaging. A single nucleus is shown for every experiment. Positions of nucleoli are indicated by "N". CB – Cajal body (marked by arrowheads). Besides binding to nuclear DNA, Draq5 also accumulates in cytoplasmic lipid drops.



Supplemental Figure S3. Co-immunoprecipitation assay using recombinant deletional PARP-1 isoforms of *Drosophila*. Purified nuclei were treated with micrococcal nuclease (MNase) to obtain complete chromatin digestion, followed by nuclear extract preparation and immunoprecipitation with anti-GFP antibody. Total proteins obtained after IP were subjected to PAGE; proteins were detected on Western blots using antibody against H4 and H2Av histones. IN – input, IP – immunoprecipitation sample, IgG – non-specific antibody control. The transcriptional factor Charon-YFP expressing animals (right) were used as a specificity control.



Supplemental Figure S4. The pre-assembled H2Av-containing core histone octamers (**A**) or mono-nucleosome (**B**) coupled to CnBr-beads were tested for interaction with PARP-1 protein deletional isoforms. The beads alone were used as a control (Mock). After binding and precipitation, proteins were subjected to PAGE and detected on Western blots using specific antibody against His-tag. IgG protein was detected on Western blot using HRB-coupled goat anti-Rabbit antibody. IN – input; P – pellet; S – supernatant. To test specificity of interactions detected in the previous experiment, we used IgG protein (negative control). IgG shows no interaction with octamer- and nucleosome-coupled beads.



Supplemental Figure S5. PARP-1 enzymatic activity assay. The full-length PARP-1 protein and W-Cat isoform are compared. Recombinant proteins were preincubated with individual co-regulator (DNA, H4, H1) or a combination of them H1-H4 and H1-DNA followed by mixing with NAD. The products of PARP-1 enzymatic reaction (poly(ADP-ribose)) were detected after PAGE on Western blots using anti-pADPr antibody.

Supplemental Table 1. Primer pairs used to detect *hsp70* DNA in ChIP experiments in Figure 2A-D.

Number	Position	5' Start	Forward	5' Start	Reverse
1	-259	4350	CTAGAATCCCAAAACAAACTGGTTG	4504	CGAGAACAGTGCGCCGTTTACTGTG
2	-231	4378	CGGTAGGTCATTTGTTTGGCAGA	4534	GAGGCGCGCTCTCTCGAAGC
3	-189	4420	CTCTGGCCGTTATTCTCTATTCGT	4571	CCTCTATTTATACTCCGGCGCTCT
4	-162	4447	GTGACTCTCCCTCTTTGTACTATTGCTC	4592	GACTCTCCGTCGACGAAGCTC
5	-134	4475	TCTCACTCTGTCACACAGTAAACGG	4625	CGATGTGTTCACTTTGCTTGTTTG
6	-103	4506	GTTCTCGTTGCTTCGAGAGAGCG	4642	GCTTAGCTTTCGCTTAGCGATG
7	-74	4535	GAATGTTCGCGAAAAGAGCG	4681	TGCAGATTGTTTAGCTTGTTCAGCT
8	-31	4578	CGTCGACGGAGAGTCAATTCTATTC	4729	CTTGGTTGTTGGTTACTTTTAATTGATTC
9	0	4609	GCAAAGTGAACACATCGCTAAGC	4770	TCTTGGTTGATTTCAGTAGTTGCAGT
10	37	4646	CAAACAAGCGCAGCTGAACAAG	4800	CAGAGTTCTCTTCTTGTCTTCAATAATTAC
11	69	4678	TGCAATAAAGTGCAAGTTAAAGTGAATC	4836	GTTCTTCTTCCTCGGTAACGACTTG
12	105	4714	AGTAACCAACAACCAAGTAATTAAACTAAA	4867	GATCGATTCCAATAGCAGGCATT
13	136	4745	CTGCAACTACTGAAATCAACCAAGAA	4903	GTTGGTAGACACCCACGCAGG
14	171	4780	GAAGACAAGAAGAGAACTCTGAATACTTTC	4935	CTGGTCGTTGGCGATAATCTCC
15	206	4815	GTCGTTACCGAGGAAGAAGAACTCA	4963	CCACGTAGGACGGCGTGGTG
16	263	4872	CACCACCTACTCCTGCGTGG	5022	GTTCATGGCCACCTGGTTCTTAG
17	329	4938	CAACCGCACCACGCCGTCCT	5097	CATGTCCTCTGCGATCTTGGG
18	391	5000	CTAAGAACCAGGTGGCCATGAAC	5145	GATCTTGGGCTTTCCGCCGT
19	459	5068	TACGACGACCCCAAGATCGC	5219	TCTTGGTCAGTACCATCGAGCTG
20	530	5139	CAAGATCGGGGTGGAGTATAAGG	5290	CGTTGAAGTAGGCTGGAACTGTG
21	594	5203	GTACTGACCAAGATGAAGGAGACGG	5355	GATGATGCGGAGCACATTCAG
22	663	5272	GTTCCAGCCTACTTCAACGACTCC	5423	GCACATTGCGCTCACCCTTG
23	732	5341	GTGCTCCGCATCATCAATGAG	5490	GAACAGTGATCCCTCGTCGATG
24	803	5412	GCGCAATGTGCTTATCTTCGAC	5562	CAGATGAGTGACTAGCCGGTTGTC
25	867	5476	GAGGGATCACTGTTCGAGGTGC	5630	CTGAGGCGTCGTAGGGCGCG
26	930	5539	GACAACCGGCTAGTCACTCATCTG	5688	CTCGATGGTGGCCTCCGTGC
27	992	5601	CCAACCCTCGCGCCCTACGA	5756	GCACAGCTCCTCAAACCTGGC
28	1059	5668	CACGGAGGCCACCATCGAGA	5823	CTTATCCATCTTGGCATCGTTGAG
29	1128	5737	GCCAGGTTTGAGGAGCTGTGC	5892	CTGCAGCAGACTTTGCACCTTG
30	1191	5800	CTCAACGATGCCAAGATGGATAAG	5956	CTCCGTATGCAACTGCCTCGT
31	1256	5865	CATTCCCAAGGTGCAAAGTCTG	6015	CAGCACGTCCTGGATCTTGC
32	1326	5935	GACGAGGCAGTTGCATACGGA	6084	CTCGATCAGCTTGGTCATTACACC
33	1391	6000	GATCCAGGACGTGCTGCTGGT	6146	GCTGGTTGTCCGCGTATGTG
34	1460	6069	GACCAAGCTGATCGAGCGCA	6224	GACAGATCGAAGGTGCCCAAT
35	1533	6142	CAGCCCGGAGTCTCCATTCA	6294	GATTCCATTGGCGTCCAAGTC

36	1604	6213	CTTCGATCTGTCCGGCATTCC	6364	GTCCCTTGTCGTTCTTGATCGTG
37	1671	6280	GACGCCAATGGAATCCTGAAC	6437	GATGCTTCTCGTCCTCGTCG
38	1739	6348	CAAGAACGACAAGGGACGGCT	6504	CTGTTCCACGGCCTGCTTCA
39	1808	6417	CGACGAGGACGAGAAGCATCG	6564	GTCGTTGCACTTGTCCAAGACG
40	1875	6484	GTGAAGCAGGCCGTGGAACA	6635	GTGAGCTCCTCCAGCTTGTGG
41	1941	6550	GACAAGTGCAACGACACTATCCG	6700	GTGCGGGAGCTGGAGCTGGT
42	2006	6615	CCACAAGCTGGAGGAGCTCAC	6765	CTCCTCGACCGTGGGTCCAG
43	2068	6677	ACCAGCTCCAGCTCCCGCAC	6822	ACCCATATGTTATAACCCATTGATGAAC
44	2135	6744	CTCTGGACCCACGGTCGAGG	6897	CTATTGTTGTTCTATGGAATATTCGATCG
45	2189	6798	CATCAATGGGTTATAACATATGGGTTATAT	6960	AATGTTATTGCTTATAGAAAAAATAAATTA
46	2252	6861	GAATGTTTCGATCGAATATTCCATAGA	7015	AGTCTACAAAACATTAAATGACAAGTTGAC
47	2317	6926	GCAAAAATGTTATTGCTTATAGAAAAAATA	7076	CATCACTAATATAGAATGTAGAATGAACCC
48	2377	6986	GTCATTTAATGTTTTGTAGACTTTTGAAAGT	7129	CAGAAAATCATCAAAGCCGAATATAAT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

***Drosophila* strains and genetics.** Genetic markers are described in FlyBase (2). Stocks were obtained from the Bloomington Stock Center, except when indicated. pP{w1, UAST::PARP-1-DsRed}, called UAS::PARP-1-DsRed, was described in (3). The transgenic stock with pP{w1, UAST::PARPe-EGFP}, herein called ZI-ZII-ZIII-A-W, was described in (4). The transgenic stock with pP{w1, UAST:: ΔPARP300-EYFP}, herein called ZII-ZIII-A-W-Cat, was described in (5). The following GAL4 driver strains were used: 69B-GAL4 (4) and Arm::GAL4 (Bloomington stock no. 1560). Balancer chromosomes carrying Kr::GFP, i.e., TM3, P{w1, Kr-GFP} and FM7i, P{w1, Kr-GFP}, were used to identify heterozygous and homozygous *parg*$^{27.1}$ and *parp*^{C03256} (4).

Construction of transgenic *Drosophila*. To make transgenic UAS::YFP constructs containing deletional isoforms of PARP-1, we generated fragments of PARP-1 cDNA corresponding to these deletions using PCR. Primers used were as follows:

ZnI-forward	CTCGAATTCATGGATATTGAATTACCTTATCTTGCT
ZnI-reverse	CTCGGTACCTGCGCTTATAACTTCTTGTATATTTTC
ZnII-forward	CAGGAATTCATGGCATTAAAAGACTTTGGGATTGAA
ZnII-reverse	CAGGGTACCCTTAGCTCTTTTTGTATCTGGTAG
ZnI-II-forward	CTCGAATTCATGGATATTGAATTACCTTATCTTGCT
ZnI-II-reverse	CAG GGT ACC GCG TTG TTT CTT TTC TCC TTC
Cat-forward	CAGGCTAGCATGTACCCAATCGAAATTCAATAT
W-Cat-forward	CAGGCTAGCATGCCAGTATCACGGACATTTAAA
A-W-Cat -forward	CAG GCT AGC ATG CCA ACA AGA TCG GCA TGC ATA GTG
ZIII-A-W-Cat -forward	CAG GCT AGC ATG GAA TTA TCA GAT ACA AAT

ZII and ZI-ZII fragments contain natural nuclear localization signals (NLS). These were cloned directly into pUAST *Drosophila* vector in frame with EYFP using EcoRI and KpnI sites. For all other constructs, pUAST polylinker was modified to allow cloning of the nuclear localization signal (NLS). Oligos used for the new polylinker were as follows:

AATTCGTTAGATCTCACGCTAGCATTACTAGTGATAGCGGCCGCGGCCTCGAGAGAGGTACCAGTT-Forward
CTAGAACTGGTACCTCTCTCGAGGCCGCGGCCGCTATCACTAGTAATGCTAGCGTGAGATCTAACG-Reverse

As a template for PCR amplification, we used plasmid LD2455, containing full-length *Drosophila* PARP-1 cDNA clone. The resulting PCR products were cloned through the *Drosophila* Gateway™ Vector Cloning System (Carnegie Institution of Washington) into the corresponding vector for *Drosophila* transformation. Transformation was performed as described (6).

Fluorescence Recovery after Photobleaching (FRAP) assay. FRAP experiments on live *Drosophila* tissues were performed as described (3). To conduct these experiments, we used a Leica TCS SP2 Confocal Microscope with capacity for FRAP. To avoid oxidative stress and other damage potentially caused by lasers, we used only the minimal level of laser power. This step extended the "bleaching" phase, but it did not affect our results. To collect FRAP data, we employed the "Fly Mode" program, which allows data collection, even during the bleaching phase. Recordings were performed by a 63× 1.4 NA oil immersion objective. We did not detect epitope-specific biases in the function, expression dynamics, or localization of any fused moiety. We used transgenic fly stocks that express the appropriate fluorescent epitope-tagged protein. Tissues were dissected in Grace's Media, and dynamic movement of fluorescent proteins was analyzed for 20-30 minutes following dissection (3). FRAP data presented in Figure 1 are based on 10 replicates.

Constructs of *Drosophila* PARP-1 and deletional isoforms for protein purification. *Drosophila* PARP-1 and deletional isoforms, including ZnI-II, ZIII-A-W-Cat, and W-Cat, were cloned from PARP-1 cDNA to exclude zinc fingers I - II, zinc fingers I - III, and zinc fingers I - automodification domain, respectively. Primers used were as follows:

PARP-1-Forward, BamHI:	5'-CAC GGA TCC ATG GAT ATT GAA TTA CCT TAT CTT GCT GAG-3'
PARP-1-Reverse, HindIII:	5'-CAC AAG CTT ATA AGA ATA CTT GAA TTC CAT ACG -3'
ZnI-II-Reverse, HindIII:	5'- CAG GGT ACC GCG TTG TTT CTT TTC TCC TTC -3'
ZIII-A-W-Cat -Forward, BamHI:	5'-CAC GGA TCC ATG GAA TTA TCA GAT ACA AAT GAA GAA GGA-3'
W-Cat -Forward, BamHI:	5'-CAC GGA TCC ATG CCA GTA TCA CGG ACA TTT AAA GTA-3'

Full-length *Drosophila* PARP-1 and deletional isoforms ZnI-II, ZIII-A-W-Cat, and W-Cat were inserted into expression vector pET-24(+) encoding a C-terminal 6-His-tag and gene of bacterial kanamycin resistance.

Recombinant protein expression, affinity purification and detection. Rosetta DE3pLysS competent cells were transformed with each respective recombinant plasmid and cultured on 0.5% glucose, kanamycin (50µg/ml), and chloramphenicol (34 µg/ml) LB plates. A 10 mL aliquot of LB with glucose and respective antibiotics was inoculated with positive colonies and shaken overnight at 37°C. A 500 mL LB glucose/ antibiotic solution was inoculated with the 10 ml sample and grown for approximately two hours at 37°C. To induce expression, 5 ml of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to the solution and incubated for three hours at the same conditions. Purification was done using Ni-column (GE Healthcare) and HPLC (GE Healthcare) according to the manufacturer's description. Detection of the respective proteins was performed after purification and Western blot assays using anti-His-tag antibody (Abcam, ab9108).

Reconstitution of mononucleosomes. H2Av histone was purified as described in (9). Core histone octamers were assembled as described in (3). Mononucleosomes were formed via "small scale" reconstitution as described in (7). ~200 bp 5S DNA obtained by PCR and purified by Qiaex II beads (Qiagen) was combined with 50 µg of each octamer per reconstitution reaction. Mononucleosomes were purified by ~3.5 ml 10-30% glycerol gradient containing 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA in 11x60 mm centrifuge tubes (Beckman). Gradients were spun at 35,000 rpm for 18 hours at 4°C. SW60 Beckman rotor was used. Mononucleosomes were quantified by ImageJ after dissociative treatment with 1 M NaCl and running on ethidium bromide-stained 0.5 x TBE 4% polyacrylamide native gels along with DNA mass ladder.

PARP-1 activity assay. 1 µl of H4-histone (1γ/µl) or endonuclease-digested plasmid DNA (0.01 γ/ µl) was mixed with 25 µl 200µM NAD and 1 µl of inhibitor/water. This mixture was combined with 10xPARP-1 reaction buffer (500 mM Tris, pH8.0, 250 mM MgCl2, 1%Triton X-100) and 0.7 µl PARP-1 enzyme (0.1γ/ µl, Trevigen). All reactions were carried out for 30 min at room temperature. Samples were examined with SDS-PAGE and Western Blot using anti-pADPr antibody.

***In vitro* Interaction Assay.** H4-histones were isolated according to the protocol of Luger et al. (7). Proteins, DNA and nucleosome coupling to CnBr-activated Sepharose beads (GE Healthcare) and *in vitro* binding assay were performed as described previously (3,9). Beads coupled to H4 histone, octamer, nucleosome or DNA were washed once for ten minutes in washing/binding buffer (10mM Tris-HCl, pH8.0, 140mM NaCl, 3mM DTT, and 0.1%Triton X-100) at room temperature with gentle rotation. Assays included a mixture of full-length *Drosophila* PARP-1 and its deletional isoforms (15 pM) in addition to mock and IgG (10 pM) controls. PARP-1 deletional isoforms and IgG were incubated with the "bait"-coupled beads in binding/ washing buffer for 20 minutes. All binding and washing were done at RT with gentle rotation. IgG interaction was detected using anti-rabbit horseradish peroxidase (1:3000, Jackson ImmunoResearch Labs), and PARP-1 deletional isoforms were detected using anti-His-tag antibody (Abcam, ab9108).

***In vitro* Interactive-Activity Assay.** After *Drosophila* PARP-1 and its deletional isoforms were bound to DNA- or histone H4-coupled Sepharose beads, as described above, the complex was washed twice with binding/washing buffer for five minutes and then incubated with activity buffer (50 mM Tris-HCl, pH 8.0, 25 mM MgCl2, 0.1% Triton X-100, and 100 µM NAD) for thirty minutes at RT with gentle rotation. Supernatant was collected, and the pellet was washed twice for five minutes with binding/washing buffer. Samples, including the supernatant, washes, and pellet, were detected after PAGE on a Western blot using anti-pADPr (10H) antibody (Calbiochem).

Western blot. The following antibodies were used for immunoblotting assays: anti-pADPr (Rabbit 1:4000, Calbiochem, #528815), anti-pADPr (Mouse monoclonal, 1:500, Tulip, #1020), anti-B-actin (Mouse monoclonal 1:5000, Sigma, #A5441), anti-His-tag antibody (Rabbit monoclonal 1:1000, Abcam, #ab9108), anti-GFP (Rabbit, Torrey Pines Biolabs, #TP401, 1:1000), and anti-GFP (Mouse monoclonal, BD, #632380, 1:5000). Western blotting was done using the detection kit from Amersham/GE Healthcare (#RPN2106), according to the manufacturer's instructions.

***Drosophila* salivary gland polytene chromosome immunostaining.** Preparation and immunostaining of polytene chromosome squashes were performed exactly as described (8). The primary antibody used was anti-GFP (Rabbit, Torrey Pines Biolabs, #TP401, 1:400), and the secondary antibody used was goat anti-rabbit Alexa-488 (Molecular Probes (1:400)). Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) with propidium iodide at 0.05 mg/ml for DNA staining.

Whole mount *Drosophila* tissues immunohistochemistry. Immunochemistry was performed as described in (9).

Mononucleosome chromatin immunoprecipitation was performed as described in (5). To detect *hsp70* DNA in ChIP experiments, we used 24 pairs of primers distributed along 3 kb of *hsp70* genomic sequence (-500 to +2500bp relative to transcriptional start) (**Supplemental Table S1**). Real-time PCR assays were performed using Power Sybr® Green PCR master mix and the StepOnePlus Real Time PCR System (Applied Biosystems). Cycling conditions were 95°C, 10 min, followed by 40 (3-step) cycles (95°C, 15 sec; 63°C, 30 sec; 72°C, 30 sec). Reactions were done in triplicate. All experiments were repeated twice, and standard deviation was calculated.

REFERENCE

1. Langelier, M. F., Planck, J. L., Roy, S. and Pascal, J. M. (2011). Crystal structures of poly (ADP-ribose) polymerase-1 (PARP-1) zinc fingers bound to DNA structural and functional insights into DNA-dependent PARP-1 activity. *J Biol Chem* 286, 10690-10701.
2. FlyBase (1999). The FlyBase database of the Drosophila genome projects and community literature. *Nucleic Acids Res.* 27, 85-88.
3. Pinnola, A., Naumova, N., Shah, M. and Tulin A.V. (2007). Nucleosomal core histones mediate dynamic regulation of PARP-1 protein binding to chromatin and induction of PARP-1 enzymatic activity. *J Biol Chem* 282, 32511-32519.
4. Tulin, A., Stewart, D. and Spradling, A.C. (2002). The Drosophila heterochromatic gene encoding poly(ADP-ribose) polymerase (PARP) is required to modulate chromatin structure during development. *Genes & Development* 16, 2108-2119.
5. Kotova, E., Jarnik, M. and Tulin, A.V. (2010). Uncoupling of the transactivation and transrepression functions of PARP-1 protein. *Proc Natl Acad Sci U S A* 107, 6406-6411.
6. Spradling, A.C. and Rubin, G.M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. *Science* 218, 341-347.
7. Luger K, Rechsteiner TJ, Richmond TJ. Expression and purification of recombinant histones and nucleosome reconstitution. *Methods Mol Biol.* 1999; 119: 1-16.
8. Tulin, A. and Spradling, A. (2003) Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at Drosophila puff loci. *Science* 299, 560-562.
9. Kotova, E., Lodhi, N., Jarnik, M., Pinnola, A.D., Ji, Y. and Tulin, A.V. (2011). Drosophila histone H2A variant (H2Av) controls poly (ADP-ribose) polymerase 1 (PARP-1) activation in chromatin. *Proc Natl Acad Sci U S A.* 108, 6205-6210.