Clickable NAD Analogues for Labeling Substrate Proteins of Poly(ADP-ribose) Polymerases

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Experimental

General methods. Reagents were obtained from Aldrich in the highest purity available and used as supplied. ^{1}H NMR and ^{13}C NMR were performed on an INOVA 500 spectrometer. LC-MS experiments were carried out on a SHIMADZU LCMS-QP8000α with a Sprite TARGA C18 column (40 × 2.1 mm, 5 μm, Higgins Analytical, Inc.) monitoring at 215 and 260 nm with positive mode for mass detection. Solvents for LC-MS were water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Rhodamine fluorescence signal from protein gel was recorded by Typhoon 9400 Variable Mode Imager (GE Healthcare Life Sciences) with setting of Green(532 nm)/555BP20 PMT450V (normal sensitivity), and analyzed by ImageQuant TL v2005. ^{32}P-NAD (800 Ci/mmol) was purchased form American Radiolabeled Chemicals, Inc. (Saint Louis, MO).

Labeling of PARP-1 truncations by PARP-1 with 6- or 8-alkyne-NAD. PARP-1 (0.15 μM), PARP-1(374-524) (4.2 μM) or PARP-1(374-1014) (2.5 μM), ssDNA (0.25 μg/μL) and 6- or 8-alkyne-NAD (100 μM) with or without NAD (100 μM) in 10 μL reaction buffer were incubated at 37 ºC for 30 min (without NAD) or 6 min (with NAD). Shorter incubation time was used because if longer incubation time is used, the protein will be extensively poly(ADP-ribosyl)ated and cannot be distinguished from the automodified PARP-1. Control experiments were done without PARP-1 full length or PARP-1 truncations. Then the click chemistry step was carried out.

Time course experiment of labeling of PARP1 (374-1014) by PARP-1 with 6-alkyne-NAD. PARP-1 (0.15 μM), PARP-1(374-1014) (2.5 μM), ssDNA (0.25 μg/μL) and 6-alkyne-NAD (100 μM) with or without NAD (100 μM) in 10 μL reaction buffer were incubated at 37 ºC for 1, 6, 30, 60 min respectively. Then the click chemistry step was carried out.

Detection limit experiment of labeling of PARP1 (374-1014) by PARP-1 with 6-alkyne-NAD. PARP-1 (0.15 μM), PARP-1(374-1014) (50 nM, 100 nM, 250 nM, 500 nM, 1μM, 2.5 μM), ssDNA (0.25 μg/μL) and 6-alkyne-NAD (100 μM) with or without NAD (100 μM) in 10 μL reaction buffer were incubated at 37 ºC for 30 min (without NAD) or 6 min (with NAD) respectively. Then the click chemistry step was carried out.

Detecting PARP-1 automodification with ^{32}P-NAD. PARP-1 (0.065 μM), ssDNA (0.25 μg/μL) and 5 μCi ^{32}P-NAD with or without cold NAD (10 μM and 100 μM) in 10 μL reaction buffer (50 mM Tris-HCl, 4 mM $MgCl_2$, 0.2 mM DTT, pH 8.0) were incubated at 37 ºC for 30 min. Then the reaction mixture was mixed with 10 μL 2× protein loading buffer and heated at 100 ºC for 6 min. The samples were then resolved by SDS-PAGE using 12% acrylamide gel. After stained with Coomassie blue and dried, the gel was exposed to a PhosphorImaging screen (GE Healthcare, Piscataway, NJ). The signal was recorded by a STORM860 phosphorimager (GE Healthcare, Piscataway, NJ).

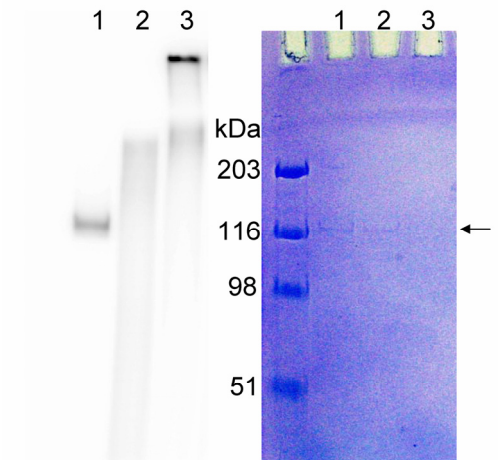


Figure S1. Labeling of PARP-1 with ^{32}P-NAD. The panel on the left shows the image of ^{32}P autoradiography. The panel on the right shows the image of the same protein gel after staining with Coomassie blue. The arrow points at the position of unmodified PARP-1. Lanes 1-3 contained PARP-1 (65 nM), ssDNA (0.25 μg/μL), and 5 μCi ^{32}P-NAD. In addition, lane 2 contained cold NAD (10 μM) and lane 3 contained cold NAD (100 μM).

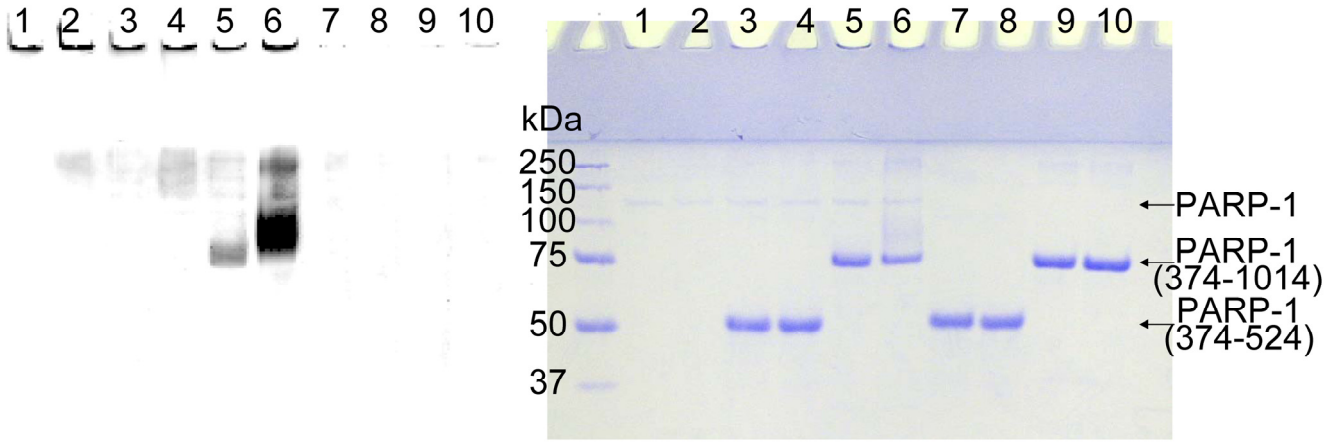


Figure S2. Labeling of PARP-1 truncations by PARP-1 using 6-alkyne-NAD. The panel on the left shows the image of Rhodamine fluorescence, and the panel on the right is the same gels stained with Coomassie blue. Lanes 1 and 2: PARP-1 (0.15 μM) with 6-alkyne-NAD (100 μM); 3 and 4: PARP-1 (0.15 μM), PARP-1(374-524) (4.2 μM) with 6-alkyne-NAD (100 μM); 5 and 6: PARP-1 (0.15 μM), PARP-1(374-1014) (2.5 μM) with 6-alkyne-NAD (100 μM); 7 and 8: PARP-1(374-524) (4.2 μM) with 6-alkyne-NAD (100 μM); 9 and 10: PARP-1(374-1014) (2.5 μM) with 6-alkyne-NAD (100 μM). There are ssDNA in all lanes, and normal NAD (100 μM) in lanes 2, 4, 6, 8, and 10.

Labeling of PARP-1 truncations by PARP-1. It has been suggested that PARP-1 auto-modification occurs across two PARP-1 molecules that form a dimer as each monomer binds to DNA.[33, 34] We wanted to test whether PARP-1 is able to modify its auto-modification domain when the domain does not bind to DNA breaks and thus cannot form a dimer with full length PARP-1. Two PARP-1 truncations were cloned, expressed in E. coli and purified. One lacks the DNA binding domain, PARP-1(374-1014), and the other contains only the auto-modification domain, PARP-1(374-524). The two PARP-1 truncations cannot be labeled with 6-alkyne-NAD when full length PARP-1 is not present (Figure S2, lanes 7-10). This is not surprising, considering that both mutants lack the DNA binding domain and thus cannot bind the ssDNA activator. In the presence of full length PARP-1, PARP-1(374-524) was weakly labeled with 6-alkyne-NAD (Figure S2, lane 3 has a stronger fluorescence than lane 1, and lane 4 has a stronger fluorescence than lane 2), while the longer truncation PARP-1 (374-1014) can be labeled very well with 6-alkyne-NAD (Figure S2, lanes 5 and 6). The smear over PARP-1 (374-1014) protein band is poly(ADP-ribosyl)ated PARP-1 (374-1014). These results demonstrate that PARP-1-catalyzed inter-molecular poly(ADP-ribosyl)ation of PARP-1 auto-modification domain can be detected with the clickable 6-alkyne-NAD.

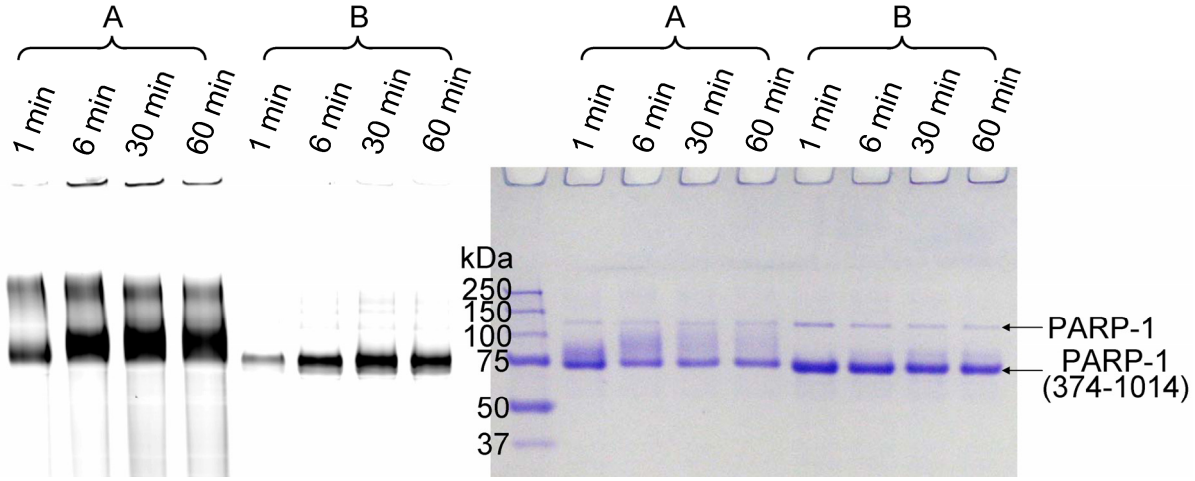


Figure S3. The time course of labeling of PARP-1(374-1014) with PARP-1 and 6-alkyne-NAD. The panel on the left shows the image of Rhodamine fluorescence, and the panel on the right is the same gels stained with Coomassie blue. PARP-1 (0.15 μM), PARP-1(374-1014) (2.5 μM), 6-alkyne-NAD

(100 μM), and ssDNA were present in labeling reactions, while extra NAD (100 μM) was only present in the reactions in lanes of **A**.

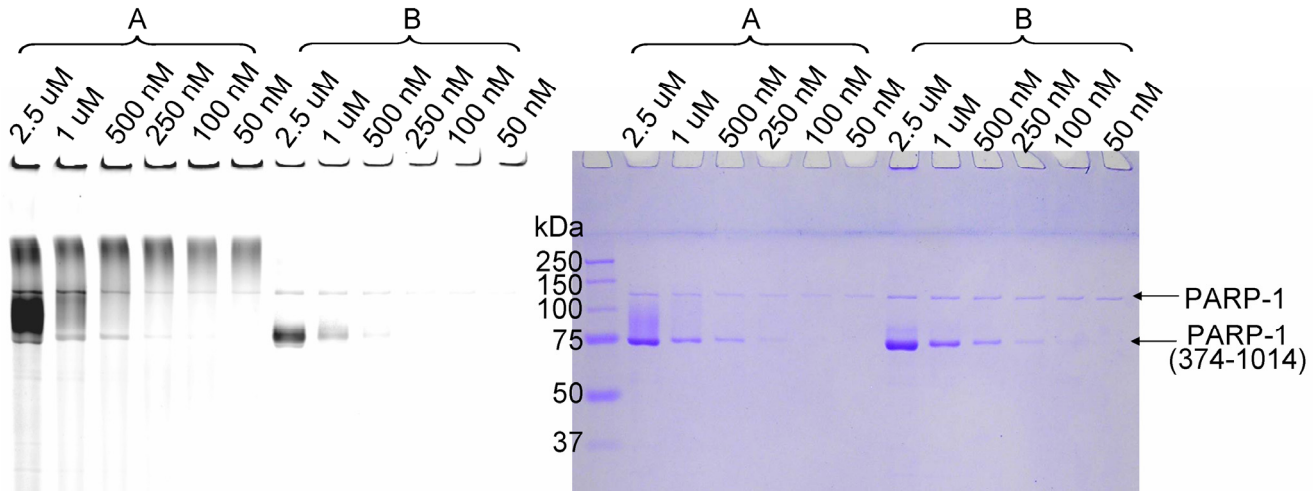


Figure S4. Detection limit experiment of labeling of PARP-1(374-1014) with PARP-1 and 6-alkyne-NAD. The panel on the left shows the image of Rhodamine fluorescence, and the panel on the right is the same gels stained with Coomassie blue. There are PARP-1 (0.15 μM), 6-alkyne-NAD (100 μM) and ssDNA in all lanes, while extra NAD (100 μM) in lanes of **A**. The reaction volumes are 10 μL and the concentration of PARP-1(374-1014) used are indicated on top of the gel images.

The time course and detection limit experiments of labeling of PARP-1(374-1014) with PARP-1 and 6-alkyne-NAD. The fact that NAD promotes the labeling reaction with 6-alkyne-NAD is further supported by the time course (Figure S3) and the detection limit of the labeling reaction (Figure S4). When labeling with both 6-alkyne-NAD and NAD, poly(ADP-ribosyl)ation of PARP-1 (374-1014) reached to maximal level around 6 min, while it took 30 min to reach maximal level when labeling with 6-alkyne-NAD only (Figure S3). In addition to a faster labeling kinetics, the labeling intensity is also higher when NAD is present in the reaction. To get an idea about the detection limit of the labeling experiment, we varied the concentration of the substrate protein, PARP-1 (374-1014). With both 6-alkyne-NAD and NAD present, 250 nmol PARP-1 (374-1014) was required to give a detectable signal (Figure S4), while 500 nmol PARP-1 (374-1014) was required when incubated with 6-alkyne-NAD only.

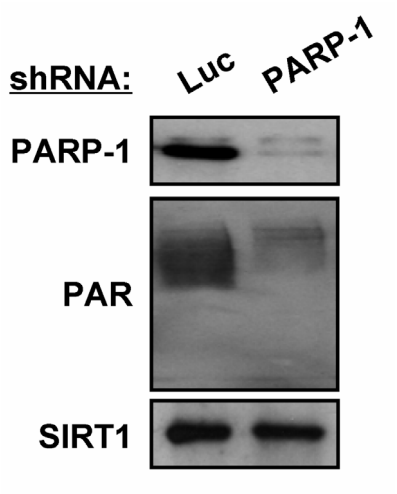
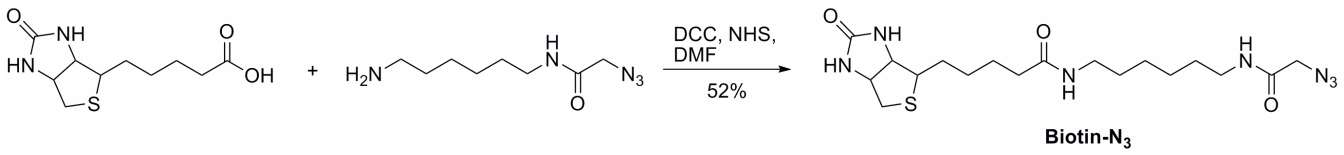


Figure S5. shRNA-mediated knockdown of PARP-1 in MCF-7 cells. Whole cell lysates collected from Luc (Luciferase, used as a control) and PARP-1 stable shRNA-mediated knockdown cell lines were subjected to Western blotting analyses for PARP-1. SIRT1 level was also analyzed as a loading control. PAR levels were analyzed by Western blotting using nuclear extracts from the same cell lines. Experiment details have been reported.[51]

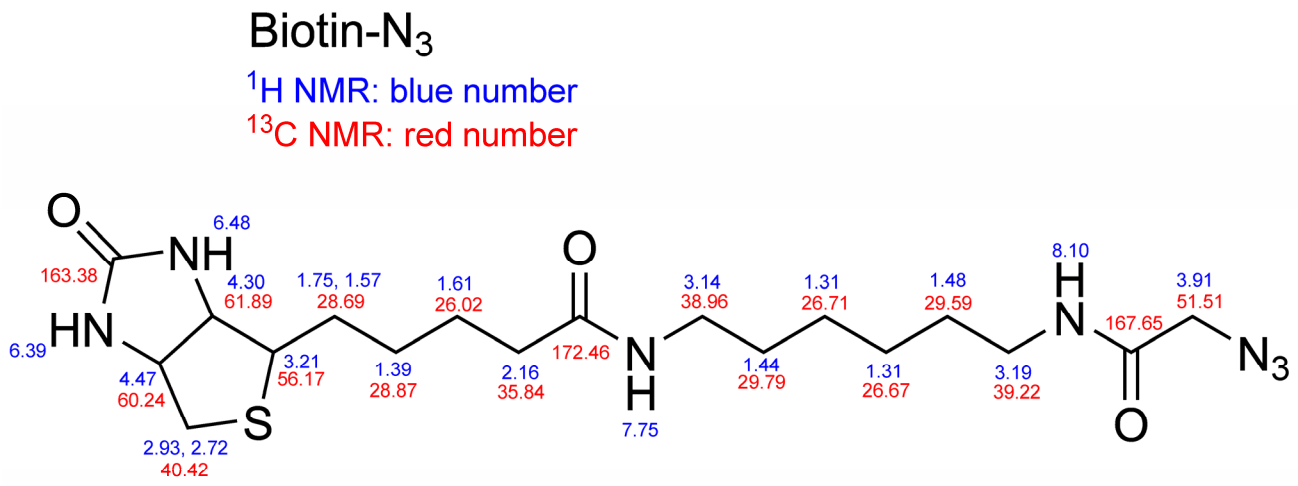
Scheme S1. Synthesis of Biotin-N_3



Synthesis of Biotin-N_3. Biotin (146.6 mg, 0.6 mmol) was added to the mixture of dicyclohexylcarbodiimide (148.6 mg, 0.72 mmol) and *N*-hydroxysuccinimide (82.9 mg, 0.72 mmol) in anhydrous DMF (2 mL) at room temperature. After stirring for 4 hours, the reaction mixture was filtered and the filtrate was added to the solution of *N*-(6-aminohexyl)-azidoacetamide (240 mg, 1.2 mmol) in anhydrous DMF (5 mL) at room temperature. The reaction was allowed to proceed at room temperature overnight. Then the precipitate was filtered and washed with CH_2Cl_2 extensively. After the

precipitate was dried *in vacuo*, the product (130 mg, 0.31 mmol, yield 52%) was used without further purification. ^{1}H NMR (500 MHz, DMF-d7): 8.10 (s, 1H), 7.75 (t, 1H, J = 7.5 Hz), 6.48 (s, 1H), 6.39 (s, 1H), 4.47 (dd, 1H, J = 10.0, 5.0 Hz), 4.30 (dd, 1H, J = 5.0, 5.0 Hz), 3.91 (s, 2H), 3.21 (m, 1H), 3.19 (m, 2H), 3.14 (dt, 2H, J = 10.0, 5.0 Hz), 2.93 (m, 1H), 2.72 (d, 1H, J = 10.0 Hz), 2.16 (t, 2H, J = 7.5 Hz), 1.75 (m, 1H), 1.61 (m, 2H), 1.57 (m, 1H), 1.48 (m, 2H), 1.44 (m, 2H), 1.39 (m, 2H), 1.31 (m, 4H). ^{13}C NMR (125 MHz, DMF-d7): 172.46, 167.65, 163.38, 61.89, 60.24, 56.17, 51.51, 40.42, 39.22, 38.96, 35.84, 29.79, 29.59, 28.87, 28.69, 26.71, 26.67, 26.02. LC-MS (ESI) calcd. for C18H32N7O3S [(M+H)$^{+}$] 426.2, obsd. 425.9.

NMR assignments for Biotin-N_3:



^{1}H NMR (500 MHz, DMF-d7): 8.10 (s, 1H), 7.75 (t, 1H, J = 7.5 Hz), 6.48 (s, 1H), 6.39 (s, 1H), 4.47 (dd, 1H, J = 10.0, 5.0 Hz), 4.30 (dd, 1H, J = 5.0, 5.0 Hz), 3.91 (s, 2H), 3.21 (m, 1H), 3.19 (m, 2H), 3.14 (dt, 2H, J = 10.0, 5.0 Hz), 2.93 (m, 1H), 2.72 (d, 1H, J = 10.0 Hz), 2.16 (t, 2H, J = 7.5 Hz), 1.75 (m, 1H), 1.61 (m, 2H), 1.57 (m, 1H), 1.48 (m, 2H), 1.44 (m, 2H), 1.39 (m, 2H), 1.31 (m, 4H).
^{13}C NMR (125 MHz, DMF-d7): 172.46, 167.65, 163.38, 61.89, 60.24, 56.17, 51.51, 40.42, 39.22, 38.96, 35.84, 29.79, 29.59, 28.87, 28.69, 26.71, 26.67, 26.02.

Table S1. Full list of potential substrate proteins of PARP-1 identified by MS using the labeling and affinity purification strategy with 6-alkyne-NAD and biotin-N_3. The emPAI ratio is emPAI in experimental samples / emPAI in negative control samples. For proteins with emPAI ratio lower than 2.00, the quantification is also confirmed by extracted ion chromatogram ratio. The extracted ion chromatogram ratio is the average of (peptide peak volume in experimental samples / peptide peak volume in negative control samples). Extracted ion chromatogram was checked for up to three common peptides found in all experimental samples and negative control samples.

Protein Accession Number		Description	MW (Da)	Known substrates of PARP-1	Potential PARP-1 substrates identified in other proteomic studies	Peptides found in samples						extracted ion chromatography ratio
	only in positive					#1	#2	#3	control #1	control #2	control #3	
GSTK1_HUMAN		Glutathione S-transferase kappa 1	25594			2	2	0	0	0	0	
HXK1_HUMAN		Hexokinase-1	103561		reported in [3]	2	0	2	0	0	0	
KU70_HUMAN		ATP-dependent DNA helicase 2 subunit 1	70084	reviewed in [1]		2	0	2	0	0	0	
MPCP_HUMAN		Phosphate carrier protein	40525		reported in [3]	2	2	0	0	0	0	
NPM_HUMAN		Nucleophosmin	32726	reviewed in [1]		2	2	0	0	0	0	
PARP1_HUMAN		Poly [ADP-ribose] polymerase 1	113811	reviewed in [1]		21	20	14	0	0	0	
RS3_HUMAN		40S ribosomal protein S3	26842		reported in [3]	4	2	2	0	0	0	
TR150_HUMAN		Thyroid hormone receptor-associated protein 3	108658			2	2	2	0	0	0	
	emPAI ratio											
SFRS1_HUMAN	6.16	Splicing factor, arginine/serine-rich 1	27842		reported in [3]	3	3	4	0	2	0	
EF1G_HUMAN	5.71	Elongation factor 1-gamma	50429			5	4	2	0	2	2	
GLYM_HUMAN	4.17	Serine hydroxymethyltransferase, mitochondrial	56414			3	2	3	0	2	0	
DDX17_HUMAN	4.11	Probable ATP-dependent RNA helicase DDX17	72953		reported in [3]	3	3	2	0	0	2	
LETM1_HUMAN	4.00	LETM1 and EF-hand domain-containing protein 1, mitochondrial	83986			3	3	2	0	3	0	
TBB2C_HUMAN	3.86	Tubulin beta-2C chain	50255		reported in [3]	6	4	5	0	4	0	
DDX5_HUMAN	3.50	Probable ATP-dependent RNA helicase DDX5	69618		reported in [3]	3	4	0	0	0	2	
SFRS6_HUMAN	3.18	Splicing factor, arginine/serine-rich 6	39677			3	0	2	2	0	0	
TMED9_HUMAN	3.15	Transmembrane emp24 domain-containing protein 9	27374			3	3	0	0	2	0	
IDH3A_HUMAN	3.00	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	40022			2	2	2	0	2	0	
RL19_HUMAN	3.00	60S ribosomal protein L19	23565		reported in [3]	2	2	2	0	2	0	
UBIQ_HUMAN	3.00	Ubiquitin	8560			2	2	2	2	0	0	
ETFA_HUMAN	2.63	Electron transfer flavoprotein subunit alpha, mitochondrial	35400			3	4	3	2	2	0	
RS24_HUMAN	2.57	40S ribosomal protein S24	15413		reported in [3]	5	0	3	2	2	0	
RBM39_HUMAN	2.55	RNA-binding protein 39	59628			2	0	3	0	0	2	
DHE3_HUMAN	2.47	Glutamate dehydrogenase 1, mitochondrial	61701		reported in [3]	5	3	6	4	0	2	
COX41_HUMAN	2.14	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	19621			3	4	3	0	2	2	
HNRPL_HUMAN	2.13	Heterogeneous nuclear ribonucleoprotein L	64720		reported in [3]	3	5	0	2	2	0	
H31T_HUMAN	2.13	Histone H3.1t	15613	reviewed in [1]		3	2	0	0	2	0	

ATP5L_HUMAN	2.00	ATP synthase subunit g, mitochondrial	11421			2	2	0	0	2	0	
HP1B3_HUMAN	2.00	Heterochromatin protein 1-binding protein 3	61454			2	2	0	0	2	0	
IMB1_HUMAN	2.00	Importin subunit beta-1	98420		reported in [3]	2	2	0	0	0	2	
MATR3_HUMAN	2.00	Matrin-3	95078		reported in [3]	2	2	0	0	2	0	
RS14_HUMAN	2.00	40S ribosomal protein S14	16434		reported in [3]	2	0	2	0	2	0	
FUS_HUMAN	1.82	RNA-binding protein FUS	53622			2	4	3	3	2	0	1.92
RL26L_HUMAN	1.81	60S ribosomal protein L26-like 1	17246		reported in [3]	4	0	3	0	2	2	1.08
SFRS7_HUMAN	1.79	Splicing factor, arginine/serine-rich 7	27578			3	2	2	2	2	0	2.12
PHB_HUMAN	1.74	Prohibitin	29843		reported in [3]	6	5	6	3	5	2	1.42
BAP31_HUMAN	1.72	B-cell receptor-associated protein 31	28031			7	4	4	2	3	4	1.29
IDHP_HUMAN	1.72	Isocitrate dehydrogenase [NADP], mitochondrial	51333			3	3	4	2	2	2	2.15
RL7_HUMAN	1.71	60S ribosomal protein L7	29264		reported in [3]	2	2	3	2	0	2	1.99
RS2_HUMAN	1.71	40S ribosomal protein S2	31590		reported in [3]	4	0	3	0	4	0	3.17
HNRPM_HUMAN	1.67	Heterogeneous nuclear ribonucleoprotein M	77749	reviewed in [1]		5	8	4	4	4	2	1.21
FUBP2_HUMAN	1.64	Far upstream element-binding protein 2	73443			5	7	3	3	4	2	1.99
H2B1C_HUMAN	1.62	Histone H2B type 1-C/E/F/G/I	13811	reviewed in [1]		6	6	4	5	4	4	3.84
CH10_HUMAN	1.60	10 kDa heat shock protein, mitochondrial	10925			4	4	3	2	3	3	1.68
QCR2_HUMAN	1.59	Cytochrome b-c1 complex subunit 2, mitochondrial	48584		reported in [3]	2	2	2	3	0	0	3.36
ANXA2_HUMAN	1.47	Annexin A2	38808		reported in [3]	8	9	6	6	7	4	1.32
HEBP1_HUMAN	1.47	Heme-binding protein 1	21198			2	0	3	0	3	0	2.35
RL15_HUMAN	1.47	60S ribosomal protein L15	24245			3	0	3	0	2	2	1.44
RS8_HUMAN	1.47	40S ribosomal protein S8	24475		reported in [3]	5	4	5	3	4	3	1.62
ECHM_HUMAN	1.46	Enoyl-CoA hydratase, mitochondrial	31823			4	5	4	2	4	2	1.72
HYOU1_HUMAN	1.45	Hypoxia up-regulated protein 1	111494			5	5	5	3	4	4	1.02
ECHB_HUMAN	1.44	Trifunctional enzyme subunit beta, mitochondrial	51547			3	6	2	2	4	2	1.06
ADT2_HUMAN	1.44	ADP/ATP translocase 2	33102		reported in [3]	6	6	4	4	4	4	2.23
ADT3_HUMAN	1.44	ADP/ATP translocase 3	33073			6	6	4	4	4	4	2.76
ROA1_HUMAN	1.43	Heterogeneous nuclear ribonucleoprotein A1	38936	reviewed in [1]		7	5	0	0	4	4	4.39
H2B1B_HUMAN	1.43	Histone H2B type 1-B	13942	reviewed in [1]		5	6	4	5	4	4	3.82
RLA0L_HUMAN	1.42	60S acidic ribosomal protein P0-like	34514			3	2	2	0	3	2	2.10
ROA3_HUMAN	1.41	Heterogeneous nuclear ribonucleoprotein A3	39799			4	4	4	3	3	3	1.92
RAB7A_HUMAN	1.41	Ras-related protein Rab-7a	23760			7	6	5	5	5	5	1.45
HNRPU_HUMAN	1.39	Heterogeneous nuclear ribonucleoprotein U	91269		reported in [3]	3	5	2	3	2	3	3.26
VDAC1_HUMAN	1.39	Voltage-dependent anion-selective channel protein 1	30868		reported in [2]	7	7	4	5	6	3	1.61
FKBP3_HUMAN	1.37	FK506-binding protein 3	25218			3	2	2	2	3	0	1.59
PYC_HUMAN	1.35	Pyruvate carboxylase, mitochondrial	130293			3	8	2	2	6	2	2.54
HNRPQ_HUMAN	1.33	Heterogeneous nuclear ribonucleoprotein Q	69788		reported in [3]	2	3	3	2	2	2	1.46
TRAP1_HUMAN	1.32	Heat shock protein 75 kDa, mitochondrial	80345			5	8	5	5	6	3	1.42
RL7A_HUMAN	1.32	60S ribosomal protein L7a	30148			6	4	5	5	3	3	1.34
RL6_HUMAN	1.31	60S ribosomal protein L6	32765		reported in [3]	5	3	2	2	3	3	1.36

ELAV1_HUMAN	1.31	ELAV-like protein 1	36240		reported in [3]	2	2	4	4	2	0	1.87
PA2G4_HUMAN	1.29	Proliferation-associated protein 2G4	44101		reported in [3]	3	5	3	4	2	2	1.19
ARF1_HUMAN	1.28	ADP-ribosylation factor 1	20741			3	0	2	0	4	0	3.21
PDIA6_HUMAN	1.26	Protein disulfide-isomerase A6	48490			9	7	6	6	7	5	1.15
FLNA_HUMAN	1.25	Filamin-A	283301		reported in [3]	3	2	0	0	2	2	No common peptide
CISY_HUMAN	1.25	Citrate synthase, mitochondrial	51908			4	3	4	4	3	2	2.51
DHB4_HUMAN	1.23	Peroxisomal multifunctional enzyme type 2	80092			5	8	6	6	5	5	1.43
RL36_HUMAN	1.22	60S ribosomal protein L36	12303			2	2	2	2	2	0	1.99
ODO2_HUMAN	1.22	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	49041			4	4	4	3	4	3	1.90
DHX9_HUMAN	1.21	ATP-dependent RNA helicase A	142181		reported in [3]	2	3	3	4	3	0	1.43
CH60_HUMAN	1.21	60 kDa heat shock protein, mitochondrial	61187		reported in [2]	19	20	16	17	17	16	1.57
NUCL_HUMAN	1.20	Nucleolin	76625	reviewed in [1]		7	8	6	5	8	5	1.53

[1] P.O. Hassa, M. O. Hottiger. The diverse biological roles of mammalian PARPs, a small but powerful family of poly-ADP-ribose polymerases. *Front. Biosci.* **2008**, *13*, 3046-3082.

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