C-BERST: Defining subnuclear proteomic landscapes at genomic elements with dCas9-APEX2

Xin D. Gao[1], Li-Chun Tu[1], Aamir Mir[1], Job Dekker[2,3,4], Scott A. Shaffer[3,5], Lihua Julie Zhu[6,7,8], Scot A. Wolfe[3,6], and Erik J. Sontheimer[1,8,*]

[1]RNA Therapeutics Institute
[2]Program in Systems Biology
[3]Department of Biochemistry and Molecular Pharmacology
[4]Howard Hughes Medical Institute
[5]Proteomics and Mass Spectrometry Facility
[6]Department of Molecular, Cell and Cancer Biology
[7]Program in Bioinformatics and Integrative Biology
[8]Program in Molecular Medicine

University of Massachusetts Medical School
Worcester, MA 01605
U.S.A.

*Correspondence: erik.sontheimer@umassmed.edu

Mapping proteomic composition at distinct genomic loci and subnuclear landmarks in living cells has been a long-standing challenge. Here we report that dCas9-APEX2 Biotinylation at genomic Elements by Restricted Spatial Tagging (C-BERST) allows the unbiased mapping of proteomes near defined genomic loci, as demonstrated for telomeres. C-BERST enables the high-throughput identification of proteins associated with specific sequences, facilitating annotation of these factors and their roles in nuclear and chromosome biology.

Three-dimensional organization of chromosomes is being defined at ever-increasing resolution through the use of Hi-C and related high-throughput methods[1]. Genome organization can also be analyzed in live cells by fluorescence imaging, especially via fluorescent protein (FP) fusions to nuclease-dead *Streptococcus pyogenes* Cas9 (dSpyCas9), which can be directed to nearly any genomic region via single-guide RNAs (sgRNAs)[2]. It has proven more difficult to map subnuclear proteomes onto 3-D genome landscapes in a comprehensive manner that avoids demanding fractionation protocols, specific DNA-associated protein fusions [e.g. in proximity-dependent biotin identification (BioID[3])], or validated antibodies. dSpyCas9 has enabled a BioID-derived subnuclear proteomic technique called CasID[4], in which biotin ligase (BirA*) fusion to dSpyCas9 allows proteins associated with specific genomic regions to be biotinylated on neighboring, exposed lysine residues in live cells. Streptavidin affinity selection and liquid chromatography/tandem mass spectrometry (LC-MS/MS) is then used to identify the tagged proteins. However, this approach is relatively inefficient and requires long (18-24h) labeling times, leading to increased background levels and limited time resolution of dynamic processes.

Engineered ascorbate peroxidase (APEX2) has been used for an alternative live-cell biotinylation strategy called spatially restricted enzymatic tagging (SRET)[5, 6]. In this approach, APEX2 is fused to a localized protein of interest, and cells are then treated with biotin-phenol and H_2O_2, generating a localized (within a ~20nm radius) burst of diffusible but rapidly quenched biotin-phenoxyl radicals. These products react with electron-rich amino acid side chains (e.g. Tyr, Trp, His and Cys), leading to covalent biotinylation of proteins in the vicinity of the localized APEX2, thus allowing subsequent identification by streptavidin selection and LC-MS/MS. Notably, this subcellular tagging method is extremely efficient (1 min H_2O_2 treatment),

allowing temporal control over the labeling process. Based in part on the success of dSpyCas9-FP fusions in enabling subnuclear imaging in living cells, we reasoned that a dSpyCas9 derivative that emits radicals rather than photons could be used for subnuclear proteomic analyses in a manner that overcomes the unfavorable kinetics and high background of CasID. Here we use dSpyCas9-APEX2 fusions in the development of C-BERST (**Fig. 1a**) for genomic element-specific profiling of subnuclear proteomes in live cells.

To develop and validate this method, we sought genomic elements that are associated with a well-defined suite of known protein factors, and that can be bound with dSpyCas9 with high efficiency and specificity using an established sgRNA. For this purpose, we chose to target telomeres in human U2OS cells. As with ~10-15% of cancer cell types, U2OS cells rely on alternative lengthening of telomeres (ALT) pathways to maintain telomere length without telomerase activation[7]. Cohorts of proteins associated with telomeres in ALT+ cells are well-characterized and map to key pathways such as homologous recombination (HR) and break-induced telomere synthesis[8]. Furthermore, an sgRNA (sgTelo) has already been established for efficient telomere association of dSpyCas9[9, 10].

We transduced U2OS cells with a lentiviral vector expressing dSpyCas9 under the control of a tet-on CMV promoter and fused to five nuclear localization signals (NLSs), a ligand-tunable degradation domain (DD)[11], mCherry, and APEX2 (**Fig. 1b**). Maximal expression of this dSpyCas9-mCherry-APEX2 fusion protein requires not only doxycycline (dox) but also the Shield1 ligand to inactivate the DD in a dose-dependent fashion[12]. This combination allows precise control over dSpyCas9-mCherry-APEX2 protein levels for optimal signal-to-noise levels. mCherry-positive cells [collected by fluorescence-activated cell sorting (FACS)] were then transduced with a separate lentiviral vector that included an sgRNA construct (driven by the U6 promoter) as well as a blue fluorescent protein (BFP) construct that also expresses the TetR repressor (**Fig. 1b**). In one version of this construct the sgRNA cassette encodes sgTelo (for labeling telomeres), and in the other it encodes a non-specific sgRNA (sgNS) that is complementary to a bacteriophage-derived sequence that is absent from the human genome[13]. After 21h of dox and Shield1 induction, we again used fluorescence-activated sorting (FACS) to sort four distinct BFP/mCherry double-positive cell populations (P1-P4) that correlate with different expression levels of dSpyCas9-mCherry-APEX and BFP (as a surrogate for sgRNA and TetR) (**Fig. 1c** and **Supplementary Fig. 1**). We reasoned that our signal-to-noise ratio of

telomeric vs. non-telomeric biotinylation would be maximized when sgTelo levels are saturating, and when dSpyCas9-mCherry-APEX2 levels are limiting (relative to potential genomic binding sites). Both conditions are expected to favor maximal partitioning of the sgRNA-programmed dSpyCas9-mCherry-APEX into the desired telomere-associated state, with as little unlocalized or mislocalized fusion protein as possible. Visual inspection by fluorescence microscopy (**Fig. 1d**) confirmed that the sgTelo P1 cell population (with higher BFP expression and lower mCherry expression) exhibited the most robust mCherry-labelled telomeric foci with the lowest amount of nucleolar or diffuse nucleoplasmic background[9]. We therefore used this population in our subsequent experiments. Diffuse nucleoplasmic labeling, as opposed to distinct foci, was observed in the sgNS P1 population (**Fig. 1d**). APEX2-catalyzed biotinylation of nucleoplasmic proteins in the sgNS control sample serves as a reference, permitting an assessment of the telomere specificity of labeling in the sgTelo sample.

We induced APEX2-catalyzed biotinylation with biotin-phenol and H_2O_2 in the sgTelo and sgNS P1 cells, and also included an sgTelo control in which the H_2O_2 was omitted. Nuclei were isolated from these cells (to reduce cytoplasmic background), and nuclear proteins were then extracted. Recovered proteins (50 µg) were subjected to western analysis using streptavidin-conjugated horseradish peroxidase (streptavidin-HRP) (**Fig. 1e**), as well as total protein visualization by Coomassie staining (**Fig. 1f**). Samples were also probed with anti-mCherry antibodies (to detect dSpyCas9-mCherry-APEX2) and HDAC1 (as a loading control) (**Fig. 1e**, bottom). Biotinylated proteins were readily detected in both sgTelo and sgNS samples, but were largely absent in the -H_2O_2 control (**Fig. 1e**). In the sgNS sample, anti-mCherry and streptavidin-HRP signals were less intense in comparison with the sgTelo sample, indicating that dSpyCas9-mCherry-APEX2 accumulation and activity are lower in the former. Biotinylated proteins were then isolated using streptavidin beads and analyzed by SDS-PAGE and silver staining (**Fig. 1g**). Aside from the ~75kDa endogenously biotinylated proteins routinely detected in SRET-labeled samples[5, 6], only background levels of proteins were detected in the no-H_2O_2 control sample, indicating successful purification. All three samples were subjected to in-gel trypsin digestion followed by LC-MS/MS to identify the biotin-labeled proteins. These analyses were done with two biological replicates prepared on different days.

The two sgTelo replicates yielded at least two unique peptides from 1,106 and 958 proteins, >80% of which (876) were detected in both. For these 876 proteins, we used intensity-

based absolute quantification (iBAQ) values to determine the degree of enrichment in the sgTelo sample relative to the sgNS sample. Some of these 876 proteins (140 in the first replicate, and 403 in the second) yielded no spectra whatsoever in the corresponding sgNS sample, consistent with sgTelo specificity. In those cases, to avoid infinitely large sgTelo/sgNS enrichment scores, we assigned those proteins the smallest non-zero iBAQ value from the proteins positively identified in that sgNS dataset. The sgTelo/sgNS iBAQ ratios were then analyzed by moderated t-test, yielding 192 proteins whose enrichment in sgTelo was statistically significant ($p < 0.05$) [**Fig. 2a** (red and blue dots) and **Supplementary Table 1**]. Strikingly, the six subunits of the shelterin complex (a telomere-binding complex that protects ends from chromosome fusion[14]) were the six most significantly enriched proteins (**Fig. 2a** and **Supplementary Table 1**). Another highly enriched protein was Apollo, a 5'→3' exonuclease that interacts with the shelterin component TRF2 and functions in the ALT pathway[15]. Overall, among the 192 most significantly sgTelo-enriched proteins (**Supplementary Table 1**), 32 have been reported previously to be associated with telomeres or linked to telomere function (**Supplementary Table 1**). These include proteins from complexes known to contribute to ALT-associated pathways or processes (**Supplementary Fig. 2**). Gene ontology (GO) analysis of the 192 C-BERST proteomic hits reveals strong functional associations with terms such as telomere maintenance, DNA replication, DNA repair, and homologous recombination, all of which are important for ALT pathways[7] (**Fig. 2b**).

Telomere-associated proteomes from ALT+ cell lines have been defined previously by TRF1-BirA* BioID in U2OS cells[16], and by biochemical purification [proteomics of isolated chromatin segments (PICh)] from WI38-VA13 cells[17]. Protein identifications from these analyses, as well as from our C-BERST dataset, were examined for overlap as depicted in the Venn diagram shown in **Fig. 2c**. Of the 192 proteins identified by C-BERST, 74 (~38%) were also detected by one or both of the other methods [62 by BioID ($p = 7.02 \times 10^{-56}$), 31 by PICh ($p = 1.58 \times 10^{-30}$), and 19 by both]. Of the 19 proteins detected by all three approaches (**Fig. 2d**), 17 are among the top third of the 192 most significant C-BERST hits based on sgTelo/sgNS enrichment, and 16 are known telomere-related factors. The remaining three of the nineteen proteins identified by PICh, BioID and now C-BERST [nitric oxide synthase-interacting protein (NOSIP), SLX4-interacting protein (SLX4IP), and core-binding factor subunit beta (CBFB) (**Fig. 2d**)] appear likely to have previously unappreciated roles as telomere-associated factors.

In conclusion, we demonstrate that C-BERST successfully maps subnuclear proteomes associated with genomic landmarks. Using the extensively investigated ALT telomeric proteome as a benchmark, we recover approximately 41% of known ALT-associated proteins (32 of 78, **Supplementary Tables 1 and 2**), as well as factors involved in all reported biological processes that contribute to ALT. By combining the flexibility of RNA-guided dSpyCas9 genome binding with the efficiency and rapid kinetics of APEX2-catalyzed biotinylation, C-BERST promises to extend the unbiased definition of subnuclear proteomes to many genomic elements, and to a range of dynamic processes (e.g. cellular differentiation, responses to extracellular stimuli, and cell cycle progression) that occur too rapidly to analyze via the 18-24h labeling procedures inherent to CasID. Importantly, C-BERST promises to augment and extend Hi-C and related methods by linking conformationally important cis-elements with the factors that associate with them. Our initial implementation of C-BERST focused on minimizing the background labeling catalyzed by un- or mis-localized dSpyCas9-mCherry-APEX2, mostly by optimizing its expression level relative to that of its sgRNA. This method is amenable to further improvements including isotope labeling[5] or tandem mass tagging[18] that will enable ratiometric assessments of differential biotinylation when dSpyCas9-mCherry-APEX2 is guided by specific vs. non-specific sgRNAs. Such improvements, in conjunction with sgRNA multiplexing, may prove to be important for applying C-BERST to the definition of subnuclear proteomes associated with non-repetitive loci. In the meantime, many types of repetitive elements within the genome, like telomeres, play critically important roles in chromosome maintenance and function in ways that depend upon associated proteins; C-BERST provides an unbiased method for sampling subnuclear, locus-specific proteomics at these elements to define protein factors critical to the functions of these elements.

METHODS

Methods, data files, and any associated references are available in the online version of the paper.

Note: Any Supplemental and Source Data files are available in the online version of the paper.

ACKNOWLEDGEMENTS

We are grateful to all members of the Sontheimer, Wolfe and Dekker labs for advice and discussions, Tom Fazzio, Samya Bhaduri and Michael Green for helpful feedback, Hanhui Ma,

David Grünwald, and Thoru Pederson for reagents, Yuehe Ding (Mello lab) for help with mass spectrometry data analysis, John Leszyk for mass spectrometry, and Lingji Zhu for assistance with figure preparation. This work was supported by 4D Nucleome grant U54 DK107980 from the National Institutes of Health to J.D., S.A.W. and E.J.S.

AUTHOR CONTRIBUTIONS

X.D.G. and E.J.S. conceived the study. X.D.G., L.-C.T., J.D., S.A.W., and E.J.S. designed experiments. X.D.G. performed the experiments. L.-C.T. processed the fluorescence images, A.M. processed flow cytometry data, and L.J.Z. conducted statistical analyses. X.D.G, L.-C.T., A.M., S.A.S., and L.J.Z. analyzed data, and all co-authors interpreted the data. X.D.G. and E.J.S wrote the manuscript, and all authors revised and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

REFERENCES

1. Davies, J.O., Oudelaar, A.M., Higgs, D.R. & Hughes, J.R. *Nat Methods* **14**, 125-134 (2017).
2. Dominguez, A.A., Lim, W.A. & Qi, L.S. *Nat Rev Mol Cell Biol* **17**, 5-15 (2016).
3. Roux, K.J., Kim, D.I., Raida, M. & Burke, B. *J Cell Biol* **196**, 801-810 (2012).
4. Schmidtmann, E., Anton, T., Rombaut, P., Herzog, F. & Leonhardt, H. *Nucleus* **7**, 476-484 (2016).
5. Hung, V. et al. *Mol Cell* **55**, 332-341 (2014).
6. Rhee, H.W. et al. *Science* **339**, 1328-1331 (2013).
7. Cesare, A.J. & Reddel, R.R. *Nat Rev Genet* **11**, 319-330 (2010).
8. Dilley, R.L. et al. *Nature* **539**, 54-58 (2016).
9. Chen, B. et al. *Cell* **155**, 1479-1491 (2013).
10. Ma, H. et al. *Proc Natl Acad Sci U S A* **112**, 3002-3007 (2015).
11. Banaszynski, L.A., Chen, L.C., Maynard-Smith, L.A., Ooi, A.G. & Wandless, T.J. *Cell* **126**, 995-1004 (2006).
12. Ma, H. et al. *J Cell Biol* **214**, 529-537 (2016).
13. Knight, S.C. et al. *Science* **350**, 823-826 (2015).
14. de Lange, T. *Genes Dev* **19**, 2100-2110 (2005).
15. Lenain, C. et al. *Curr Biol* **16**, 1303-1310 (2006).
16. Garcia-Exposito, L. et al. *Cell Rep* **17**, 1858-1871 (2016).
17. Dejardin, J. & Kingston, R.E. *Cell* **136**, 175-186 (2009).
18. Loh, K.H. et al. *Cell* **166**, 1295-1307 e1221 (2016).

ONLINE METHODS

Construction of C-BERST plasmids. The Shield1- and doxycycline-inducible dSpyCas9-mCherry-APEX2 construct was made by subcloning Flag-APEX2 from Flag-APEX2-NES (Addgene 49386) into DD-dSpyCas9-mCherry[12] using the pHAGE backbone. Two NLSs (SV40 and nucleoplasmin NLS) were inserted at each terminus to improve nuclear localization. The sequence of the final plasmid is provided in the **Supplementary Note**. The sgTelo-encoding construct was created by replacing the C3-guide RNA sequence (pCMV_C3-sgRNA_2XBroccoli/pPGK_TetR_P2A_BFP) with sgTelo sequences (using a plasmid provided by Hanhui Ma and Thoru Pederson). Non-specific sgRNA (sgNS)[13] was used similarly to create the analogous sgNS construct.

Cell culture and cell line construction. Human U2OS cells obtained from Thoru Pederson's lab (originally obtained from ATCC) were cultured in Dulbecco-modified Eagle's Minimum Essential Medium (DMEM; Life Technologies) supplemented with 10% (vol/vol) FBS (Sigma). Lentiviral transduction was as described[12]. Six-fold higher titers of sgRNA-encoding lentiviruses were used for transduction relative to dSpyCas9-APEX2 lentivirus.

Flow cytometry. One day before performing FACS, dox (Sigma; 2 μg/ml) and Shield1 (Clontech; 250 nM) were added to the media. Cells expressing dSpyCas9-mCherry-APEX2 and BFP sgRNA were selected by FACSAria cell sorter or analyzed with MacsQuant® VYB. Both instruments are equipped with 405- and 561-nm excitation lasers, and the emission signals were detected by using filters at 450/50 nm (wavelength/bandwidth) for BFP, and 610/20 nm (FACSAria) or 615/20nm (MacsQuant) for mCherry. Bulk population and single cells (**Supplementary Fig. 1b**) were sorted into plates containing 1% GlutaMAX, 20% FBS, and 1% penicillin/streptomycin in DMEM medium.

Fluorescence microscopy. U2OS cells expressing sgRNA were seeded onto 170 μm, 35 × 10 mm glass-bottom dishes (Eppendorf) supplemented with dox and Shield1 21 hours before imaging. Live cells were imaged with a Leica DMi8 microscope equipped with a Hamamatsu camera (C11440-22CU), a 63x oil objective lens, and Microsystems software (LASX). Further

imaging processing was done with MetaMorph (Molecular Devices). Image contrast was set to ease visualization of cell, foci and nucleoplasmic background.

C-BERST biotinylation protocol. Six 15cm plates of U2OS cells expressing sgTelo or sgNS were used in this assay. Dox (2 µg/ml) and Shield1 (250 nM) were added 21 hours before biotinylation. Cells were then incubated with 500 µM biotin-phenol (BP) (Adipogen) for 30 minutes at 37°C. 1 mM H_2O_2 was then added to initiate of biotinylation for 1 minute on a horizontal shaker at room temperature. Six 15cm plates of sgTelo-expressing cells were treated in parallel, but without H_2O_2 addition, as a negative control. Quencher solution (5 mM trolox, 10 mM sodium ascorbate, and 10 mM sodium azide) was added to stop the reaction, and cells were washed five times (three quencher washes and two DPBS washes) to continue the quench and to remove excess BP.

Enrichment of biotinylated proteins. Cells were scraped off the plates and used for the preparation of isolated nuclei[19]. Nuclei were washed with DPBS before lysis. RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.125% SDS, 0.125% sodium deoxycholate and 1% Triton X-100 in Millipore water) with 1x freshly supplemented Halt Protease Inhibitor were used to lyse the cells for 10 minutes on ice. Cell lysates in 1.5 ml Eppendorf tubes were sonicated for 15 minutes with a Diagenode Bioruptor with 30s on/off cycles at high intensity. Cell lysates were clarified by centrifugation at 13,000 rpm for 10 minutes. Clarified protein samples (~3.5 mg) were subjected to 400 µl Dynabeads MyOne Streptavidin T1 affinity purification overnight at 4°C. Each bead sample was washed with a series of buffers to remove non-specifically bound proteins: twice with RIPA lysis buffer, once with 1 M KCl, once with 0.1 M Na_2CO_3, once with 2 M urea in 10mM Tris-HCl, pH 8.0, and twice with RIPA lysis buffer. Proteins were eluted in 70 µl 3x protein loading buffer supplemented with 2 mM biotin and 20 mM DTT with heating for 10min at 95˚C[5]. 50 µl eluents were loaded and run on a 4-12% SDS-PAGE gel (Bio-Rad) and run approximately 1cm off the loading well for in-gel digestion and LC-MS/MS analysis.

Western blotting. Protein concentrations of the cell lysates were determined by BCA assay (Thermo). 50 ug of each sample was mixed with protein loading buffer, boiled, and separated in

SDS-PAGE gels. Proteins were transferred to PVDF membrane (Millipore), and blotted with Streptavidin-HRP (Thermo), or with anti-mCherry (Abcam) or anti-HDAC1 (Bethyl) antibodies.

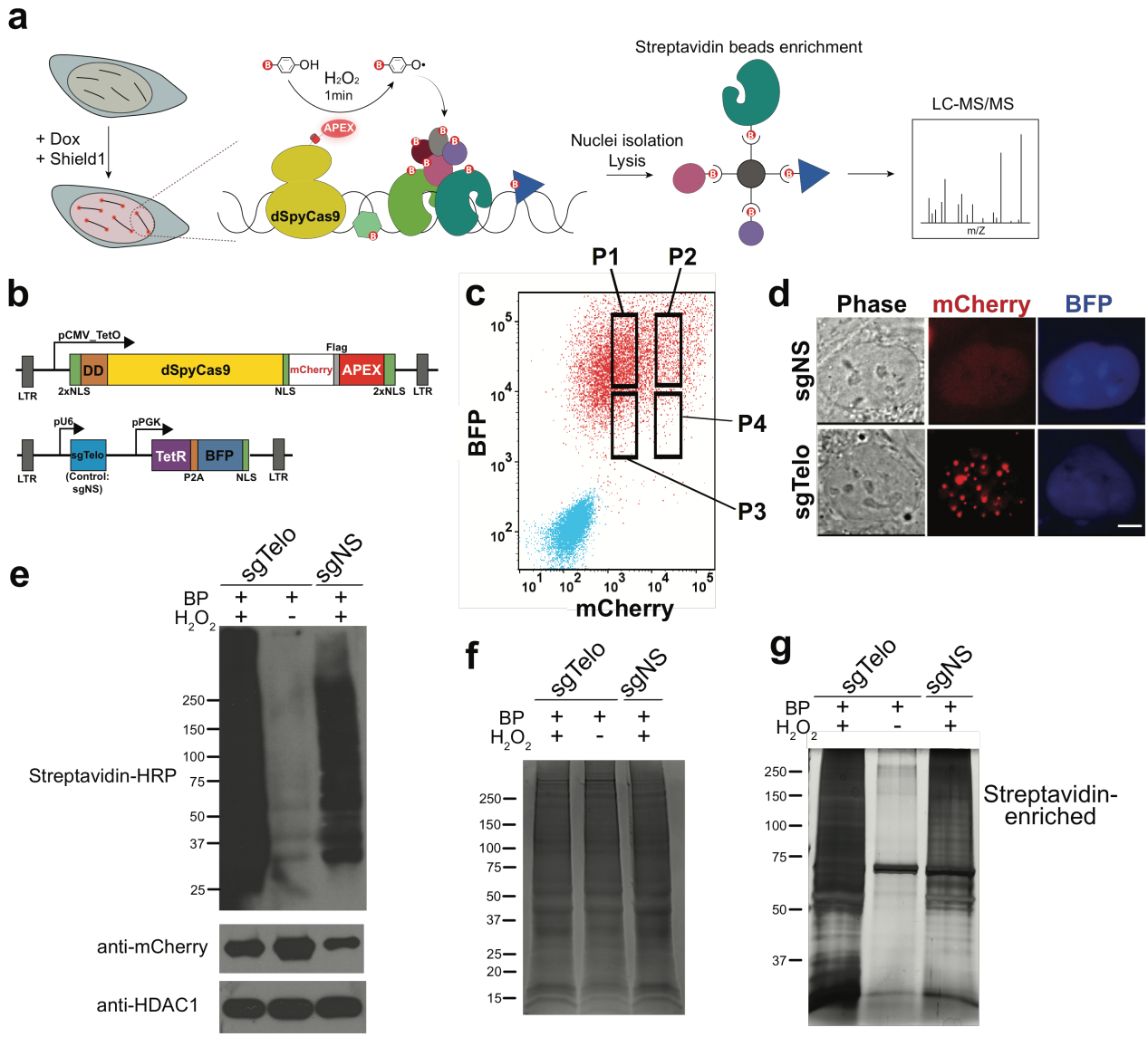
LC-MS/MS and proteomic analyses. Unresolved protein bands from SDS-PAGE were cut into 1x1 mm pieces and placed in 1.5ml Eppendorf tubes with 1ml of water. After 30 min, water was removed and replaced with 70 µl of 250 mM ammonium bicarbonate. Proteins were then reduced by the addition of 20 µl of 45 mM 1,4-dithiothreitol, incubated at 50˚C for 30 min, cooled to room temperature, alkylated with 20 µl of 100 mM iodoacetamide for 30 min, and washed twice with 1 ml water. The water was removed and replaced with 1 ml of 50 mM ammonium bicarbonate: acetonitrile (1:1) and incubated at room temperature for 1 hr. The solvent was then replaced with 200 µl acetonitrile, removed, and the pieces dried in a Speed Vac. Gel pieces were then rehydrated in 75 µl of 4 ng/µl sequencing-grade trypsin (Promega) in 0.01% ProteaseMAX Surfactant (Promega) in 50 mM ammonium bicarbonate and incubated at 37˚C for 21 hr. The supernatant was then removed to a 1.5 ml Eppendorf tube, the gel pieces further dehydrated with 100 µl of acetonitrile: 1% (v/v) formic acid (4:1), and the combined supernatants dried on a Speed Vac. Peptides were then reconstituted in 25 µl of 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid for LC-MS/MS.

Samples were analyzed on a NanoAcquity UPLC (Waters Corporation) coupled to a Q Exactive (Thermo Fisher Scientific) hybrid mass spectrometer. In brief, 1.0 µl aliquots were loaded at 4 µl/min onto a custom-packed fused silica precolumn (100 µm ID) with Kasil frit containing 2 cm Magic C18AQ (5µ, 100Å) particles (Bruker Corporation). Peptides were then separated on a 75µm ID fused silica analytical column containing 25 cm Magic C18AQ (3µm, 100Å) particles (Bruker) packed in-house into a gravity-pulled tip. Peptides were eluted at 300 nl/min with a linear gradient from 95% solvent A (0.1% (v/v) formic acid in water) to 35% solvent B (0.1% (v/v) formic acid in acetonitrile) in 60 min. Data was acquired by data-dependent acquisition according to a published method[20]. Briefly, MS scans were acquired from *m/z* 300-1750 at a resolution of 70,000 (*m/z* 200) and followed by ten tandem mass spectrometry scans using HCD fragmentation using an isolation width of 1.6 Da, a collision energy of 27%, and a resolution of 17,500 (*m/z* 200). Raw data files were processed with Proteome Discoverer (Thermo, version 2.1.1.21) and searched with Mascot (Matrix Science, version 2.6) against the SwissProt *Homo*

sapiens database. Search parameters used tryptic specificity considering up to 2 missed cleavages, a parent mass tolerance of 10 ppm, and a fragment mass tolerance of 0.05 Da. Fixed modification of carbamidomethyl cysteine was considered as were variable modifications of N-terminal acetylation, N-terminal conversion of Gln to pyroGlu, oxidation of methionine, and biotin-phenol conjugation of tyrosine. Results were loaded into Scaffold (Proteome Software Inc., version 4.8.2) for peptide and protein validation and quantitation using the Peptide Prophet and Protein Prophet algorithms[21, 22]. The threshold for peptides was set to 70% (1.1% FDR) and 90% for proteins (2-peptide minimum). Contaminants such as human keratin were included in all statistical analyses and removed from the figures.

Data analysis. Data was first filtered to exclude proteins detected in only one of the S1 replicates, followed by log2 transformation. Prior to the $\log_2$ transformation, iBAQ values of 0 were replaced with the smallest iBAQ value from the corresponding sample in S2 or S3 to avoid generation of infinite ratios. Moderated t-test with a paired design was used to compare the log2 transformed iBAQ values between S1 and S3, S1 and S2, and S2 and S3 using limma package[23]. To adjust for multiple comparisons, *p* values were adjusted using the Benjamini-Hochberg (BH) method[24]. Proteins with BH-adjusted *p* values less than 0.05 are considered statistically significant. Proteins were selected for subsequent GO (David Bioinformatics) and overlap analysis if they were (*i*) significantly enriched in both S1 vs. S3 and S1 vs. S2, (*ii*) not enriched in S2 vs. S3, and (*iii*) if S1/S3 and S1/S2 ratios were greater than 2. To determine whether the proteins identified in this experiment overlap significantly with two published datasets, a hypergeometric test was used.

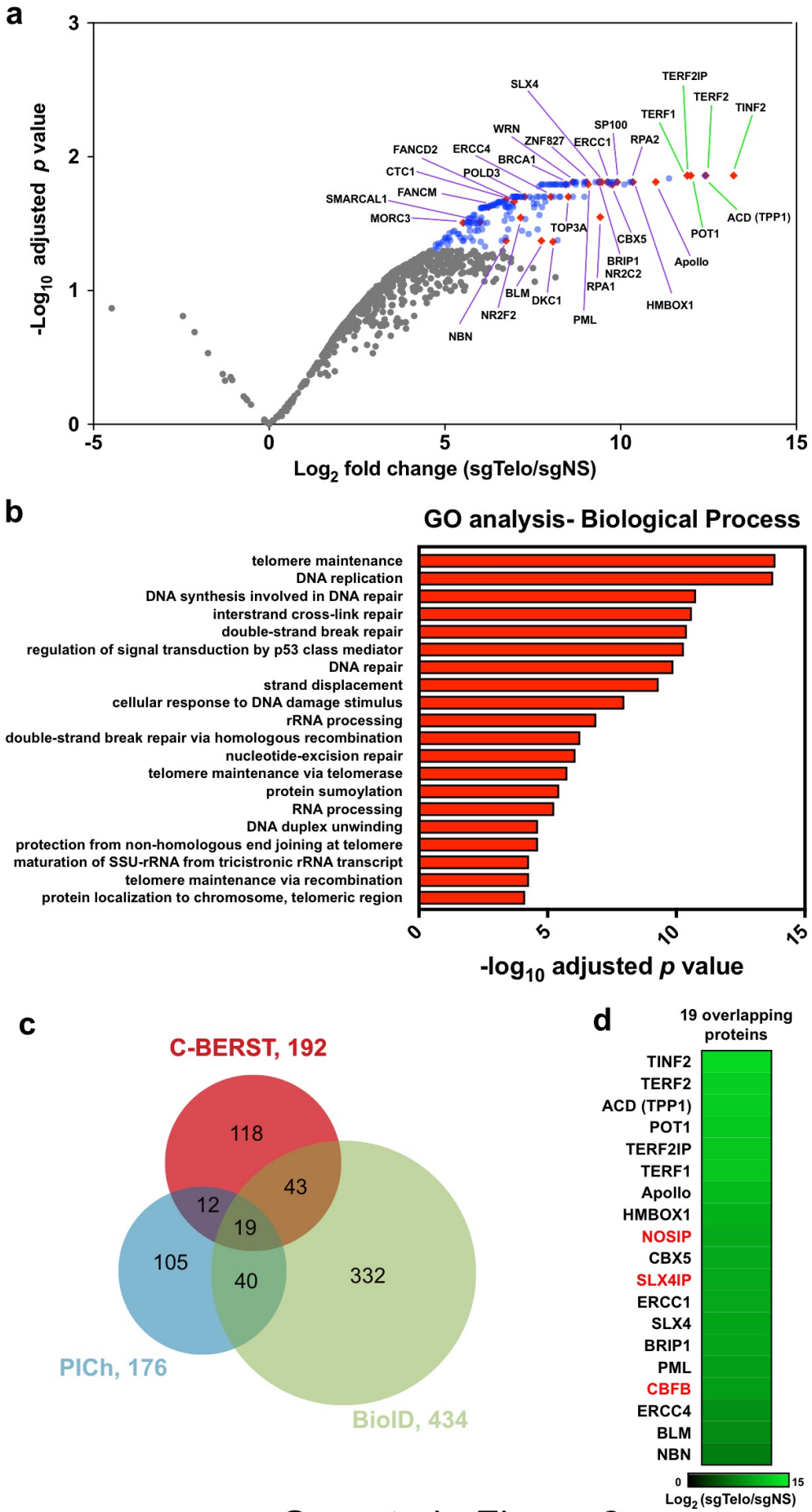
19. Nagano, T. et al. *Nature* **502**, 59-64 (2013).
20. Kelstrup, C.D. et al. *J Proteome Res* **13**, 6187-6195 (2014).
21. Keller, A., Nesvizhskii, A.I., Kolker, E. & Aebersold, R. *Anal Chem* **74**, 5383-5392 (2002).
22. Nesvizhskii, A.I., Keller, A., Kolker, E. & Aebersold, R. *Anal Chem* **75**, 4646-4658 (2003).
23. Smyth, G.K. *Stat Appl Genet Molec Biol* **3**, 1-25 (2004).
24. Benjamini, Y. & Hochberg, Y. *J R Stat Soc B* **57**, 289-300 (1995).



Gao et al., Figure 1

357

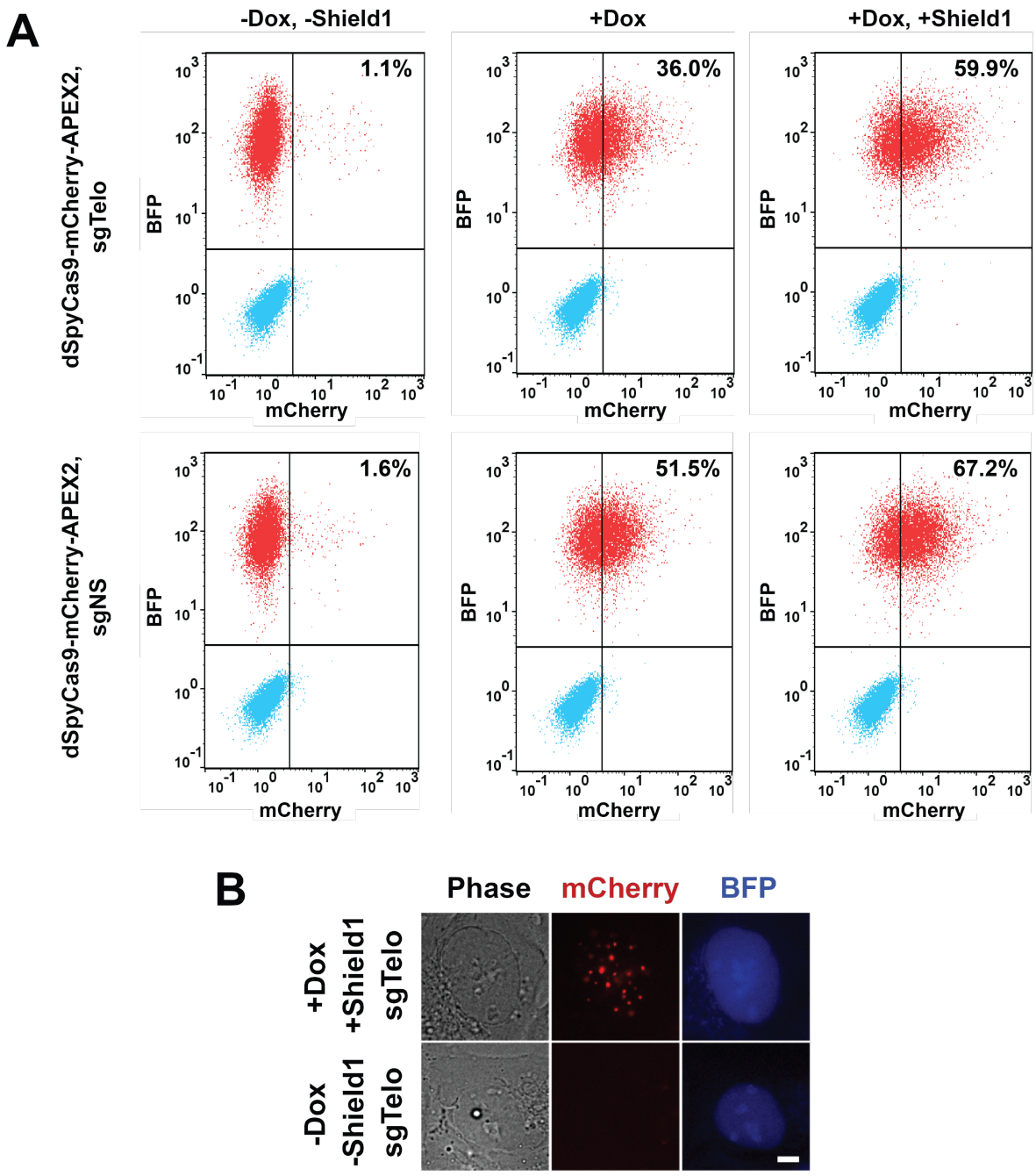
Figure 1 Using C-BERST to biotinylate telomere-associated proteins in living human cells. (**a**) Diagram of the C-BERST workflow. U2OS cells stably expressing sgRNA and inducible dSpyCas9-APEX2 are generated by lentiviral transduction. Following dox and Shield1 induction (21 h), cells are incubated with biotin-phenol (BP, 30 min) and then H_2O_2 (1 min) to activate a burst of biotin-phenoxyl radical generation by dSpyCas9-APEX2, leading to proximity-labeling of nearby proteins. Following quenching, nuclei isolation and protein extraction, biotinylated proteins are enriched by streptavidin selection and analyzed by LC-MS/MS. (**b**) The dSpyCas9-mCherry-APEX2 and sgRNA lentiviral expression constructs. Top: dSpyCas9-mCherry-APEX2 under the control of the pCMV_TetO inducible promoter. The mCherry fusion is included to enable quantification of dSpyCas9 expression level as well as its subcellular localization. NLS, nuclear localization signal; LTR, long terminal repeat; DD, Shield1-repressible degradation domain. Bottom: sgRNA/TetR/BFP expression construct. pU6, U6 promoter; pPGK, PGK promoter; sgTelo, telomere-targeting sgRNA; sgNS, non-specific sgRNA; tetR, tet repressor; P2A, 2A self-cleaving peptide; BFP, blue fluorescent protein. (**c**) FACS sorting of untransduced (blue) and mCherry- and BFP-positive cells (red). The P1 population corresponds to high BFP (as a surrogate for sgRNA and TetR) and low mCherry expression, providing optimal signal-to-noise ratio to maximize the fraction of telomere-localized dSpyCas9-mCherry-APEX2. (**d**) Live-cell imaging of telomere localization by dSpyCas9-mCherry-APEX2 in U2OS cells, using the P1-sorted population defined in (**c**). dSpyCas9-mCherry-APEX2 exhibited telomeric foci with sgTelo but not with sgNS. Scale bar, 5 μm. (**e**) Top: Western blot analysis of dSpyCas9-mCherry-APEX2 biotinylation, as detected by streptavidin-HRP. sgRNAs, BP treatment, and H_2O_2 treatment are indicated at the top of each lane. Anti-mCherry was used to detect dSpyCas9-mCherry-APEX2 (middle), and anti-HDAC1 was used as a loading control (bottom). (**f**) Coomassie-stained SDS-PAGE of total protein from isolated nuclei following biotin labeling. (**g**) Silver-stained SDS-PAGE of biotin-labeled proteins enriched with streptavidin-coated beads. In **e-g**, the mobilities of protein markers (in kDa) are indicated on the left of each panel.



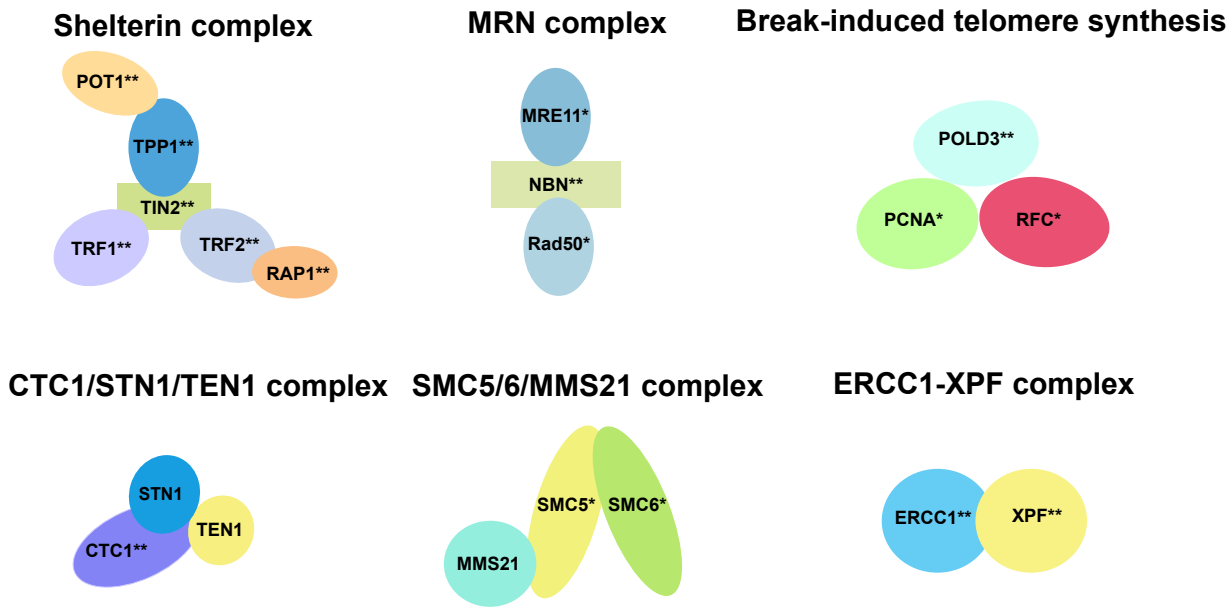
384

Gao et al., Figure 2

Figure 2 Successful capture of telomere-associated proteins in living human cells by C-BERST. (**a**) Volcano plot of C-BERST-labeled, telomere-associated proteins in U2OS cells. Intensity-based absolute quantification (iBAQ) values from the MS analyses were calculated for each identified protein for all three samples (sgTelo + H_2O_2, sgTelo - H_2O_2, and sgNS + H_2O_2). 192 proteins (indicated by blue and red) are statistically enriched [Benjamini-Hochberg (BH)-adjusted p value < 0.05] in the sgTelo + H_2O_2 sample, relative to both control samples. The 32 proteins indicated by red diamonds (with identities provided) are previously defined as either telomere-associated proteins or ALT pathway components. These include all six shelterin components (indicated by green lines). (**b**) Gene Ontology-Biological Process (GO-BP) analysis on the 192 telomeric/ALT proteins identified by C-BERST. The x-axis is the -$\log_{10}$ p value (BH-adjusted) for the C-BERST-detected proteins associated with each GO term given on the left. The 20 most statistically significant GO terms are displayed. (**c**) Venn diagram of statistically enriched (BH-adjusted p value < 0.05) telomeric proteins from ALT human cells, as detected by C-BERST (red), PICh (purple), and TRF1-BirA* BioID (green). 74 proteins from the C-BERST proteome were also detected by PICh, BioID, or both. (**d**) A heat map of the C-BERST $\log_2$ fold-change enrichment scores for the 19 telomeric proteins identified by all three proteomic approaches from (**c**). All 19 proteins are highly enriched in the C-BERST telomere proteome. The three proteins indicated in red have not been reported previously as telomere- or ALT-associated.



Supplementary Fig. 1 Inducible dSpyCas9-mCherry-APEX2 expression. (**a**) Flow cytometry was used to measure the percentage of mCherry+ and BFP+ double-positive cells under different induction conditions. Stable U2OS cells expressing sgTelo (top row) or sgNS (bottom row) were exposed to three conditions for 21h before flow cytometry: no inducers (left), dox only (2 μg/ml, middle), or a combination of dox (2 μg/ml) and Shield1 (250 nM) (right). Cyan: untransduced cells; red: transduced cells. With both sgRNAs, dox and Shield1 in combination yield the highest percentages of double-positive cells. Specific percentages of mCherry+, BFP+ cells are indicated in each plot. (**b**) Live-cell imaging of clonal cells derived from the sgTelo P1 population (see **Fig. 1c**). When inducers are omitted, dSpyCas9-mCherry-APEX2 expression and telomeric accumulation are not observed. Scale bar, 5 μm.



Supplementary Fig. 2 C-BERST specifically detects components of multiple complexes and factors implicated in ALT pathways. Proteins denoted by double asterisks were significantly enriched (BH-adjusted p value < 0.05) in the sgTelo + H_2O_2 sample, relative to the sgTelo - H_2O_2 and sgNS + H_2O_2 samples (see **Supplementary Table 1**). Proteins denoted by single asterisks were also detected but with lower degrees of enrichment (BH-adjusted p value > 0.05) in the sgTelo + H_2O_2 sample (**Supplementary Table 1**). Components of the RAD9/RAD1/HUS1 complex (see **Supplementary Table 2**) were not detected.

Supplementary Note dSpyCas9-mCherry-APEX2, sgTelo, and sgNS sequences.

Amino acid sequence of dSpyCas9-mCherry-APEX2

Legend: NLS DD dSpyCas9 *mCherry* FLAG APEX2

MAPKKKRKVEDKRPAATKKAGQAKKKKEDACGVQVETISPGDGRTFPKRGQTCVVHYTGMLE
DGKKVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPH
ATLVFDVELLKPEGSEFGSGSDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKK
NLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEED
KKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNP
DNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLI
ALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRV
NTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFY
KFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREK
IEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPN
EKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDY
FKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERL
KTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDD
SLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMAR
ENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQEL
DINRLSDYDVDAIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLI
TQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVIT
LKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKM
IAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL
SMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEK
GKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASA
GELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVIL
ADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLI
HQSITGLYETRIDLSQLGGDGSTSGSPKKKRKVGSGS*MVSKGEEDNMAIIKEFMRFKVHMEGS*
VNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLK
LSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEA
SSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYT
*IVEQYERAEGRHSTGGMDELYK*SGGRGGGGSDYKDDDDKGKSYPTVSADYQDAVEKAKKKL
RGFIAEKRCAPLMLRLAFHSAGTFDKGTKTGGPFGTIKHPAELAHSANNGLDIAVRLLEPLKAEF
PILSYADFYQLAGVVAVEVTGGPKVPFHPGREDKPEPPPEGRLPDPTKGSDHLRDVFGKAMGL
TDQDIVALSGGHTIGAAHKERSGFEGPWTSNPLIFDNSYFTELLSGEKEGLLQLPSDKALLSDP
VFRPLVDKYAADEDAFFADYAEAHQKLSELGFADALEPKKKRKVEDKRPAATKKAGQAKKKKG
S*

Nucleotide sequence of sgRNA/TetR_P2P_BFP

Legend: *U6 promoter* Guide sequence sgRNA scaffold *hPGK promoter* TetR P2A BFP NLS

ccccttcaccgagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgta
aacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactat
*catatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccg*NNNNNNNNNNNNNNNNNNNNtttaagagctatgctggaaacagcatagcaagtttaaataaggctagtccgttatcaacttgaaaaagtggcaccgag
tcggtgctttttttgaattctcgacctcgagacaaatggcagtattcatccacaattttaaaagaaaaggggggattgggggtacagtgc
aggggaaagaatagtagacataatagcaacagacatacaaactaaagaattacaaaaacaaattacaaaaattcaaaattttcgg
gtttattacagggacagcagagatccactttggccgcggctcgaggggg*ttggggttgcgccttttccaaggcagccctgggtttgcgc*
agggacgcggctgctctgggcgtggttccgggaaacgcagcggcgccgaccctgggtctcgcacattcttcacgtccgttcgcagcg
tcacccggatcttcgccgctacccttgtgggccccccggcgacgcttcctgctccgcccctaagtcgggaaggttccttgcggttcgcgg
cgtgccggacgtgacaaacggaagccgcacgtctcactagtaccctcgcagacggacagcgccagggagcaatggcagcgcgc
cgaccgcgatgggctgtggccaatagcggctgctcagcagggcgcgccgagagcagcggccgggaaggggcggtgcgggagg
cggggtgtggggcggtagtgtgggccctgttcctgcccgcgcggtgttccgcattctgcaagcctccggagcgcacgtcggcagtcgg
*ctccctcgttgaccgaatcaccgacctctctccccag*ggggatccgtgagtttggggaccttgattgttctttcttttctgctattgtaaaatt
catgttatatggaggggcaaagttttcagggtgttgtttagaatgggaagatgtcccttgtatcaccatggaccctcatgataatttgtttc
tttcactttctactctgttgacaaccattgtctcctcttattttcttttcattttctgtaacttttcgttaaactttagcttgcatttgtaacgaatttttaaa
ttcacttttgtttatttgtcagattgtaagtactttctctaatcacttttttttcaaggcaatcagggtatattatattgtacttcagcacagttttagag
aacaattgttataattaaatgataaggtagaatatttctgcatataaattctggctggcgtggaaatattcttattggtagaaacaactacat
cctggtcatcatcctgcctttctctttatggttacaatgatatacactgtttgagatgaggataaaatactctgagtccaaaccgggcccctc
tgctaaccatgttcatgccttcttcttttttcctacagctcctgggcaacgtgctggttattgtgctgtctcatcattttggcaaagaattgtaatac
gactcactatagggcgaattgatatgtctagattagataaaagtaaagtgattaacagcgcattagagctgcttaatgaggtcggaatc
gaaggtttaacaacccgtaaactcgcccagaagctaggtgtagagcagcctacattgtattggcatgtaaaaaataagcgggctttgc
tcgacgccttagccattgagatgttagataggcaccatactcacttttgccctttagaaggggaaagctggcaagatttttttacgtaataa
cgctaaaagttttagatgtgctttactaagtcatcgcgatggagcaaaagtacatttaggtacacggcctacagaaaaacagtatgaaa
ctctcgaaaatcaattagccttttttatgccaacaaggtttttcactagagaatgcattatatgcactcagcgctgtggggcattttactttagg
ttgcgtattggaagatcaagagcatcaagtcgctaaagaagaaagggaaacacctactactgatagtatgccgccattattacgaca
agctatcgaattatttgatcaccaaggtgcagagccagccttcttattcggccttgaattgatcatatgcggattagaaaaacaacttaaa
tgtgaaagtgggtccgcgtacagcggctcccgggagttcgctagcggtgctactaacttcagcctgctgaagcaggctggagacgtg
gaggagaaccctggacctggtagtggaacgcgtatggtgtctaagggcgaagagctgattaaggagaacatgcacatgaagctgt
acatggagggcaccgtggacaaccatcacttcaagtgcacatccgagggcgaaggcaagccctacgagggcacccagaccatg
agaatcaaggtggtcgagggcggccctcccccttcgccttcgacatcctggctactagcttcctctacggcagcaagaccttcatcaa
ccacacccagggcatccccgacttcttcaagcagtccttccctgagggcttcacatgggagagagtcaccacatacgaagatgggg
gcgtgctgaccgctacccaggacaccagcctccaggacggctgcctcatctacaacgtcaagatcagaggggtgaacttcacatcc
aacggccctgtgatgcagaagaaaacactcggctgggaggccttcaccgagacgctgtaccccgctgacggcggcctggaaggc
agaaacgacatggccctgaagctcgtgggcgggagccatctgatcgcaaacgccaagaccacatatagatccaagaaacccgct
aagaacctcaagatgcctggcatctactatgtggactacagactggaaagaatcaaggaggccaacaacgaaacctacgtcgagc
agcacgaggtggcagtggccagatactgcgacctccctagcaaactggggcacaagcttaatccaaagaaaaagcggaaagtg

Guide sequences:

sgTelo: gTTAGGGTTAGGGTTAGGGTT

sgNS: gAATCTCGCTTATATAACGAG