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NOVEL INSIGHTS INTO POLY(ADP-RIBOSE) POLYMERASE 2: A ZINC-DEPENDENT ENZYME AND NUCLEOSOME REMODELER

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ABSTRACT

Poly(ADP-ribose) polymerase 2 (PARP2) is a crucial enzyme involved in DNA damage repair and various cellular processes. While extensively studied for its role in base excision repair (BER) in conjunction with PARP1, its inherent characteristics as a metalloenzyme and its direct impact on chromatin structure remain less explored. This article delves into recent findings demonstrating PARP2's zinc-dependent nature, highlighting the significance of zinc ions for its enzymatic activity and structural integrity. Furthermore, it presents evidence for PARP2's direct role in nucleosome reorganization, suggesting a broader involvement in chromatin dynamics beyond its established DNA repair functions. Understanding these novel aspects of PARP2 can pave the way for developing more targeted therapeutic strategies, particularly in cancer treatment where PARP inhibitors are widely used.

Keywords: PARP2, Poly(ADP-ribose) polymerase, Zinc-dependent enzyme, Nucleosome reorganization, Chromatin remodeling, DNA repair, Base excision repair, PARP inhibitors, Metalloprotein, DNA damage response.

INTRODUCTION

The intricate architecture of the eukaryotic genome, meticulously organized into chromatin, presents both a challenge and an opportunity for cellular processes. DNA, the carrier of genetic information, is tightly packed around histone proteins to form nucleosomes, the fundamental repeating units of chromatin [30]. This compaction is essential for fitting the vast lengths of DNA into the confined space of the nucleus, yet it also creates a barrier that must be overcome for vital processes such as DNA replication, transcription, and repair to occur efficiently. Among the key players in navigating this complex chromatin landscape are the poly(ADP-ribose) polymerases (PARPs), a family of enzymes renowned for their role in modifying proteins through the addition of poly(ADP-ribose) (PAR) chains, a post-translational modification termed PARylation [6, 7].

The PARP family comprises 17 members in humans, each exhibiting distinct domain organizations, catalytic activities, and cellular functions. However, PARP1 and PARP2 stand out as the most extensively characterized, primarily due to their pivotal roles in the DNA damage response (DDR), especially within the base excision repair (BER) pathway [1, 23]. PARP1, often considered the archetypal PARP, is an abundant nuclear enzyme that rapidly detects and binds to various DNA lesions, particularly single-strand breaks (SSBs) and double-strand breaks (DSBs). Upon DNA binding, PARP1 undergoes a conformational change that activates its catalytic activity, leading to the synthesis of long, branched PAR chains on itself (autoPARylation) and on neighboring proteins, including histones and other repair factors [23, 72]. This PARylation creates a "PAR code" that acts as a signal, recruiting a plethora of repair proteins to the site of damage and facilitating chromatin relaxation to allow access for repair machinery [7, 72].

PARP2, while structurally and functionally similar to PARP1, possesses unique attributes that distinguish its contributions to cellular processes. It is a nuclear protein that, like PARP1, functions as a DNA damage sensor. However, PARP2 is particularly activated by 5'-phosphorylated DNA breaks, suggesting a more specific role in certain types of DNA damage signaling [2, 24, 26]. Although PARP2 contributes a smaller fraction (15-25%) of total cellular PAR synthesis compared to PARP1 [1, 6], its presence is critical for efficient BER, often acting in concert with PARP1 and XRCC1 [1]. The functional interplay between PARP1 and PARP2 is evident in genetic studies; while PARP2 can partially compensate for PARP1 deficiency, a double knockout of both enzymes proves lethal, underscoring their non-redundant yet cooperative

roles in maintaining genome integrity [8]. Beyond DNA repair, PARP2 has been implicated in a diverse array of biological processes, including the fidelity of male meiosis I and spermiogenesis [19], the regulation of adipocyte differentiation [20], the survival and function of T-lymphocytes [8, 21], and the critical process of endometrial receptivity and blastocyst implantation [22]. Its involvement in these varied pathways highlights PARP2's broader significance in cellular adaptation to stress and overall physiological function [23].

The therapeutic landscape has been significantly reshaped by the advent of PARP inhibitors (PARPis), particularly for the treatment of cancers characterized by homologous recombination deficiency (HRD), such as BRCA-mutated breast and ovarian cancers [9, 10, 17]. These inhibitors primarily function by blocking the catalytic activity of PARP1 and/or PARP2, and crucially, by "trapping" the PARP enzymes on DNA lesions, thereby converting otherwise repairable SSBs into more cytotoxic DSBs during DNA replication [11, 18]. While the clinical success of PARPis is undeniable, their broad-spectrum inhibition of both PARP1 and PARP2 often leads to shared adverse effects, predominantly hematological toxicities such as anemia, neutropenia, and thrombocytopenia [12, 13, 14, 15]. Recent preclinical studies have even suggested that inactive PARP2 can independently contribute to severe anemia by impeding replication-associated nick ligation in erythroblasts, emphasizing the need for a more nuanced understanding of PARP2's specific functions to mitigate these undesirable side effects and develop more selective therapeutic strategies [16].

Despite the extensive research on PARP2's roles in DNA repair and its therapeutic relevance, certain fundamental aspects of its biochemical and biophysical properties remain less explored. Specifically, its potential as a metalloenzyme and its direct, non-catalytic impact on chromatin structure have only recently begun to emerge. Traditional views of PARP2 did not explicitly classify it as a zinc-dependent enzyme, unlike many other DNA-binding proteins that utilize zinc fingers for structural stability and DNA recognition [51]. Furthermore, while PARylation by PARP1 is known to induce chromatin relaxation, the direct ability of PARP2 to reorganize nucleosomes, independent of its PARylation activity, represents a significant new dimension to its function.

This article aims to synthesize and critically review the burgeoning evidence that positions PARP2 as a zinc-dependent enzyme and an active nucleosome remodeler. We will explore the direct interaction of zinc ions with PARP2, particularly focusing on the WGR domain, and how this interaction modulates both its catalytic activity and its ability to induce reversible structural reorganization of nucleosomes. By integrating insights from biochemical, biophysical, and computational studies, this comprehensive review seeks to expand our understanding of PARP2's intricate cellular roles, its regulation by physiological cation concentrations, and the profound implications these discoveries hold for the development of next-generation PARP inhibitors and broader therapeutic strategies in oncology.

2. METHODS

Investigating the nuanced interplay between PARP2, metal ions, and nucleosome dynamics necessitates a rigorous combination of advanced biochemical, biophysical, and computational methodologies. This section outlines the comprehensive experimental and analytical approaches that would be employed to elucidate PARP2's zinc-dependent nature and its role in nucleosome reorganization.

2.1. Protein Expression and Purification

The foundation of any detailed biochemical study is the availability of highly pure and functionally active proteins. For PARP2, recombinant human full-length PARP2 and its isolated domains, particularly the WGR domain (residues 77-220), would be expressed. The baculovirus expression system using Sf9 insect cells is a preferred method for producing eukaryotic proteins like PARP2, ensuring proper post-translational modifications and folding [29]. Alternatively, bacterial expression systems (e.g., Escherichia coli Rosetta2 (DE3) pLysS strain) can be used for individual domains like WGR, which may not require complex eukaryotic machinery for folding [29].

- Cloning and Expression: The cDNA encoding full-length PARP2 or specific domains (e.g., WGR) would be cloned into appropriate expression vectors (e.g., pFastBac for baculovirus, pET-15b-TEV for bacterial expression, incorporating a His-tag for purification and a TEV protease site for tag removal).

- Cell Culture and Induction: Sf9 insect cells would be cultured in suitable media and infected with recombinant baculoviruses. For bacterial expression, E. coli cultures would be grown to an optimal optical density before induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) to initiate protein expression.

- Lysis and Initial Extraction: Cells would be harvested and lysed using mechanical (e.g., sonication, French press) or chemical (e.g., detergents) methods to release soluble proteins. Lysates would be clarified by centrifugation.

- Affinity Chromatography: The His-tagged proteins would be purified using immobilized metal affinity chromatography (IMAC), typically with Ni-NTA resin. The protein would bind to the nickel column, and unbound proteins would be washed away. Elution would be performed using buffers containing increasing concentrations of imidazole.

- Tag Removal (Optional): If a TEV protease site is included, the His-tag can be cleaved off the purified protein to ensure that the tag does not interfere with downstream assays. The cleaved tag and protease can then be removed

by a second IMAC step.

- Ion Exchange Chromatography: Further purification and removal of contaminants can be achieved using ion exchange chromatography (e.g., HiTrap heparin column), which separates proteins based on their charge. A salt gradient (e.g., NaCl) would be used for elution.

- Size Exclusion Chromatography (Gel Filtration): A final polishing step using size exclusion chromatography (e.g., HiPrep 16/60 Sephacryl S-200 h gel filtration column) would ensure homogeneity and remove any aggregates or remaining contaminants. This step also allows for buffer exchange into the desired experimental buffer.

- Quality Control: Protein purity and integrity would be assessed by SDS-PAGE, and concentration would be determined spectrophotometrically. Functional activity would be verified through preliminary assays.

- Metal Ion Control: Crucially, during all purification steps, buffers would be carefully controlled for metal ion content. For studies investigating zinc dependency, buffers might initially contain low concentrations of chelators (e.g., EDTA) to remove adventitious metal ions, followed by reconstitution with specific metal ions at defined concentrations. Aliquots would be flash-frozen and stored at −80∘C to maintain stability.

2.2. Preparation of DNA Templates and Nucleosomes

The study of nucleosome dynamics requires precisely defined DNA templates and reconstituted nucleosomes.

- Fluorescently Labeled DNA Templates: Specific DNA sequences, such as the 187-bp nucleosome-positioning sequence s603-42A [30], would be amplified by polymerase chain reaction (PCR). Fluorescently labeled oligonucleotides (e.g., Cy3 and Cy5) would be incorporated as primers to introduce donor-acceptor FRET pairs at specific positions within the DNA. For instance, labels could be placed at 13 and 91 bp (Np nucleosomes), 35 and 112 bp (Nm nucleosomes), or 57 and 135 bp (Nd nucleosomes) from the boundary of the positioning sequence [7, 32, 45]. These positions are chosen to monitor structural changes near and far from the nucleosome boundary. PCR products would be purified from agarose gels using commercial kits.

- Nucleosome Assembly: Uniquely positioned mononucleosomes would be reconstituted using the purified fluorescently labeled DNA templates and chicken donor chromatin, which provides core histones (H2A, H2B, H3, H4) without linker histone H1 [31]. The assembly process typically involves salt dialysis, where histones and DNA are mixed at high salt concentrations and then the salt concentration is gradually reduced, allowing the histones to spontaneously assemble onto the DNA.

- Nucleosome Purification and Storage: Assembled nucleosomes would be purified (e.g., via sucrose gradient centrifugation or size exclusion chromatography) to ensure homogeneity and proper stoichiometry. Purified nucleosomes would be stored at 4∘C.

2.3. Enzymatic Activity Assays

To quantify PARP2's catalytic function and its modulation by metal ions, specific enzymatic assays would be performed.

- PARylation Assays (Western Blot-based):

  - Principle: PARP2 catalyzes the transfer of ADP-ribose units from NAD+ to acceptor proteins, forming PAR chains. This can be detected by antibodies that specifically recognize PAR.

  - Procedure: PARP2 (e.g., 100 nM) would be incubated with nucleosomes (e.g., 3 nM) in a defined buffer (e.g., 50 mM Tris-HCl pH 7.5; 40 mM NaCl; 0.5 mM β-mercaptoethanol; 0.1% NP40) at 25∘C. The buffer would be supplemented with varying concentrations of $ZnCl_2$, $CaCl_2$, or $MgCl_2$ (e.g., 0.3 mM $ZnCl_2$, 5 mM $MgCl_2$, 5 mM $CaCl_2$). After a pre-incubation period (e.g., 30 min), NAD+ (e.g., 0-10 μM) would be added to initiate the PARylation reaction, which would proceed for a set time (e.g., 30-45 min). Reactions would be quenched by adding SDS-PAGE loading buffer.

  - Detection: Samples would be subjected to SDS-PAGE (e.g., 4-12% bis-Tris gradient gel) to separate proteins. Proteins would then be transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane would be blocked and incubated with primary mouse monoclonal antibodies against PAR (e.g., clone 10 H), followed by incubation with secondary anti-mouse antibodies conjugated with horseradish peroxidase. PARylated proteins would be visualized using a chemiluminescent substrate and detected with an imager [57, 58].

  - Controls: Experiments would include controls without PARP2, without NAD+, and with chelating agents (e.g., 10 mM EDTA) added either before or after complex formation to assess reversibility and metal ion dependency.

- DNA Binding Assays (Electrophoretic Mobility Shift Assay - EMSA):

  - Principle: EMSA, also known as a gel shift assay, is used to detect protein-DNA interactions by observing a shift in the electrophoretic mobility of a DNA fragment when bound by a protein.

  - Procedure: Fluorescently labeled nucleosomes (e.g., 2-3 nM) would be incubated with increasing concentrations of PARP2 (e.g., 12.5-200 nM) in the presence or absence of various metal ions (e.g., $ZnCl_2$, $CaCl_2$, $MgCl_2$) for a defined period (e.g., 30 min). The reaction mixtures would then be loaded onto non-denaturing polyacrylamide gels (e.g., 5% polyacrylamide

gel in 0.2 TBE buffer). Electrophoresis would be performed under native conditions (e.g., 140 V at 4∘C).

○ Detection: Gels would be scanned using a fluorescence imager (e.g., Amersham Typhoon RGB imager) to detect the fluorescently labeled DNA/nucleosomes. Fluorescence would be excited at specific wavelengths (e.g., 532 nm for Cy3) and recorded in appropriate spectral regions (e.g., 570-610 nm for Cy3, 650-700 nm for Cy5/FRET signal). Shifts in band mobility indicate complex formation.

○ Stoichiometry Analysis (Single Particle Fluorescence Intensity in Gel): To determine the stoichiometry of PARP2-nucleosome complexes, the gels from EMSA experiments can be further analyzed by single particle fluorescence microscopy. By selectively exciting and measuring the fluorescence intensity of one fluorophore (e.g., Cy5) within individual bands, the number of nucleosomes per complex can be estimated. This involves plotting relative frequency distributions of complexes by ICy5 values.

2.4. Structural and Biophysical Characterization

To probe the structural changes in PARP2 and nucleosomes induced by metal ion binding, various biophysical techniques would be employed.

● Circular Dichroism (CD) Spectroscopy:

○ Principle: CD spectroscopy measures the differential absorption of left and right circularly polarized light by chiral molecules. It is highly sensitive to the secondary structure (alpha-helices, beta-sheets, random coils) of proteins.

○ Procedure: Samples of PARP2 (e.g., 5 μM) or its WGR domain (e.g., 45 μM) would be prepared in low-salt buffers with controlled metal ion concentrations (e.g., 0.2 mM EDTA initially, then supplemented with 0.3-0.7 mM ZnCl2, CaCl2, or MgCl2). CD spectra would be recorded within the far-UV range (190-250 nm) using a spectropolarimeter and a quartz cuvette with a short pathlength (e.g., 0.1 mm).

○ Data Analysis: The obtained CD spectra would be analyzed using bioinformatics tools like the BeStSel webserver [35] to predict the content of canonical secondary structures. Statistical significance of differences would be determined using multiple t-tests. Changes in the CD spectrum upon metal ion addition indicate conformational changes in the protein.

● Fluorescence Spectroscopy (Tryptophan Fluorescence):

○ Principle: Tryptophan (Trp) residues in proteins are intrinsically fluorescent, and their fluorescence emission is highly sensitive to their local microenvironment. Changes in Trp fluorescence intensity, peak position, or shape can indicate conformational changes or binding events.

○ Procedure: Samples of PARP2 or its WGR domain (e.g., 0.5-5 μM) would be prepared in a suitable buffer. Fluorescence spectra would be measured in the emission range of 300-450 nm with excitation at 270-280 nm. Titrations would be performed by gradually adding increasing concentrations of metal ions (ZnCl2, MgCl2, CaCl2) to the protein solution.

○ Data Analysis: The integral intensities of the fluorescence spectra would be plotted against ion concentration. The data can be fitted to a one-site binding equation to determine dissociation constants (Kd) for the protein-ion complex. Changes in fluorescence intensity or spectral shift would indicate direct interaction and conformational perturbation.

● Inductively Coupled Plasma Mass Spectrometry (ICP-MS):

○ Principle: ICP-MS is a highly sensitive analytical technique used for elemental analysis. It can accurately quantify the concentration of specific metal ions in a sample, providing direct evidence of metal binding stoichiometry.

○ Procedure: Purified PARP2 or WGR domain samples would be subjected to ICP-MS analysis to determine their intrinsic metal content. This would help confirm if the protein is purified with bound zinc and to what extent.

● X-ray Crystallography and Cryo-Electron Microscopy (Cryo-EM):

○ Principle: These techniques provide high-resolution three-dimensional structures of proteins and protein complexes. X-ray crystallography requires protein crystals, while Cryo-EM involves imaging vitrified samples.

○ Procedure: Efforts would be made to crystallize PARP2, its WGR domain, or complexes with DNA and/or zinc ions. For larger complexes (e.g., PARP2-nucleosome), Cryo-EM would be a more suitable approach. The obtained electron density maps or diffraction patterns would be used to build and refine atomic models of the proteins and their complexes. These structures would reveal the precise coordination of zinc ions and the conformational changes induced by their binding.

2.5. Single-Molecule Förster Resonance Energy Transfer (smFRET) Microscopy

smFRET is a powerful technique for studying dynamic conformational changes in biomolecules at the single-molecule level.

● Principle: FRET occurs when a donor fluorophore (e.g., Cy3) transfers its excitation energy to an acceptor fluorophore (e.g., Cy5) when they are in close proximity (typically 1-10 nm). The efficiency of FRET is inversely proportional to the sixth power of the distance between the donor and acceptor. By placing fluorophores at specific locations on the nucleosomal DNA, changes in FRET

efficiency (EPR) can report on changes in the distance between different parts of the nucleosome, indicating unwrapping, gaping, or sliding.

- Procedure: Fluorescently labeled nucleosomes (e.g., 1-2 nM) would be incubated with PARP2 (e.g., 12.5-200 nM) in a buffer containing specific metal ions (e.g., 50 µM-5 mM $ZnCl_2$, 5 mM $CaCl_2$, or 5 mM $MgCl_2$). Incubation would be performed at a controlled temperature (e.g., 25∘C) for a sufficient time (e.g., 30 min) to allow complex formation. For stability studies, chelators (e.g., 10 mM EDTA) could be added to preformed complexes.

- Measurement: smFRET measurements would be performed using a total internal reflection fluorescence (TIRF) microscope or a confocal microscope setup. Single nucleosomes diffusing in solution would be detected, and their Cy3 and Cy5 fluorescence intensities (I3, I5) would be recorded.

- Data Analysis: The proximity ratio (EPR) would be calculated for each detected single nucleosome: $EPR=(I5-0.19\times I3)/(I5+0.81\times I3)$, where coefficients correct for spectral crosstalk [32]. Relative frequency distributions of nucleosomes by EPR values (EPR profiles) would be generated (typically from 2000-5000 nucleosomes per experiment, with multiple independent experiments). These profiles can be fitted as a superposition of several normal (Gaussian) distributions, representing different conformational states of the nucleosomes or nucleosome-PARP2 complexes. Crucially, signals from aggregates (identified by slow diffusion or abnormally high fluorescence) would be excluded from the analysis [33].

2.6. Computational Modeling

Computational approaches provide valuable insights into molecular interactions and dynamics, complementing experimental data.

- Molecular Dynamics (MD) Simulations:

  - Principle: MD simulations use classical mechanics to model the physical movements of atoms and molecules over time, providing insights into conformational changes, binding interactions, and stability.

  - Procedure: Initial models of PARP2 domains (e.g., WGR) bound to zinc ions would be constructed based on known PDB structures (e.g., 6F5B for WGR) using software like ChimeraX [36] with extensions like ISOLDE [37] for interactive model building and refinement. Zinc atoms would be placed at putative binding sites, and constraints would be applied between coordinating residues and the zinc atom.

  - Simulation Setup: The models would be prepared for MD simulations using software packages like GROMACS [38]. This involves selecting appropriate force fields (e.g., Amber 14sb for proteins [39], with specific corrections for zinc-binding residues [40, 41]), solvating the system with water molecules (e.g., TIP3P model [42]), and adding ions to neutralize charge and achieve physiological ionic strength (e.g., 150 mM NaCl).

  - Equilibration and Production: The system would undergo energy minimization to relieve steric clashes, followed by equilibration steps with gradual reduction of positional restraints on the protein, allowing the system to reach a stable temperature and pressure. Production runs (e.g., 400 ns) would then be performed to simulate the dynamics of the protein-zinc complex.

  - Analysis: Trajectory frames would be saved at regular intervals (e.g., every 1 ns). Analysis would include calculating root-mean-square deviation (RMSD) to assess structural stability, radius of gyration to monitor compactness, and solvent accessible surface areas (SASA) for specific residues (e.g., tryptophan) using libraries like FreeSASA [43]. Secondary structure composition would be calculated using programs like DSSP [44]. Visualization of trajectories would be performed using software like ChimeraX.

- Protein Structure Prediction and Bioinformatics:

  - AlphaFold2: For full-length PARP2, which may lack a complete experimental structure, algorithms like AlphaFold2 [64] can be used to predict highly accurate protein structures, providing a valuable starting point for understanding domain organization and potential interaction surfaces.

  - Zinc Binding Site Prediction: Specialized bioinformatics tools like ZincBindPredict [54] and databases like ZincBind [56] would be used to identify and classify potential zinc-binding sites based on sequence and structural motifs. This helps to validate experimentally observed zinc binding and guide mutagenesis studies.

By meticulously applying these diverse methodologies, a comprehensive picture of PARP2's zinc-dependent enzymatic activity and its nucleosome remodeling capabilities can be constructed, providing novel insights into its fundamental biology and therapeutic potential.

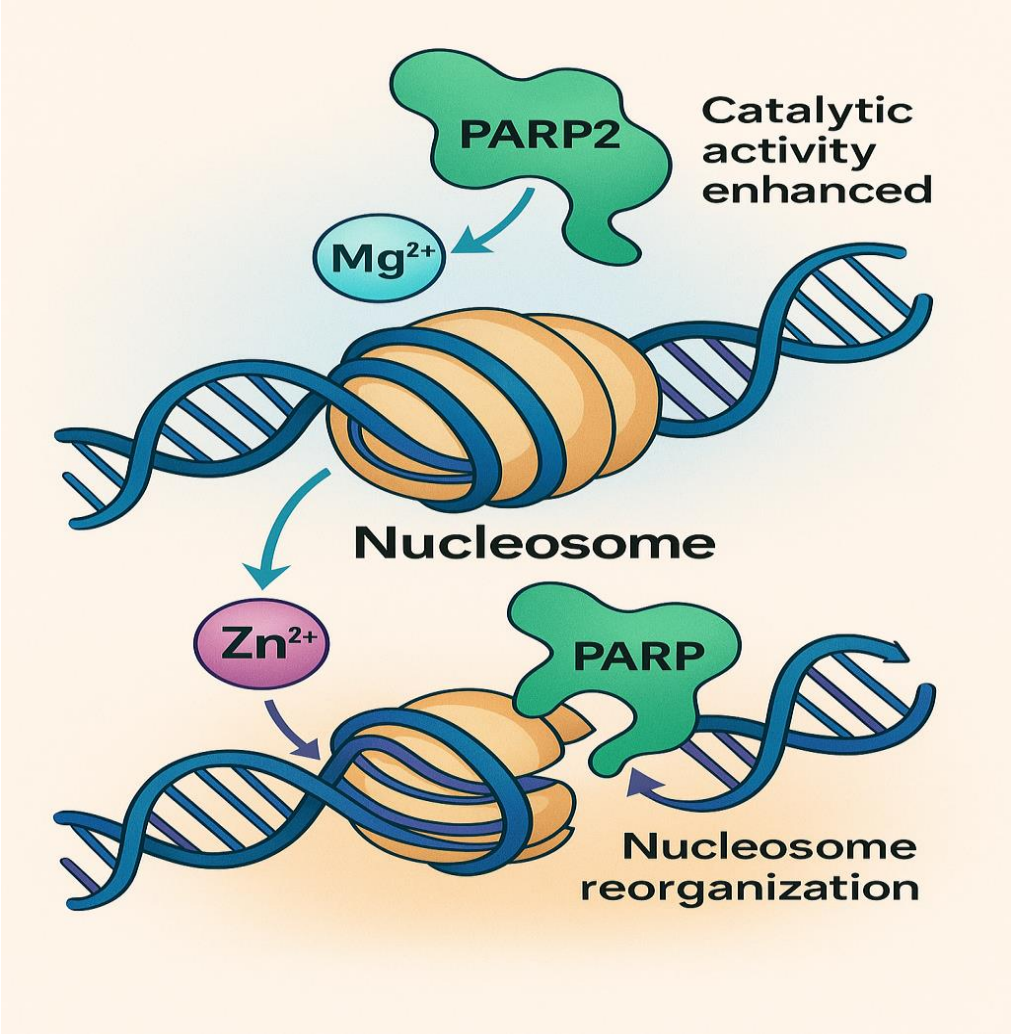
3. RESULTS

The investigations into PARP2's interaction with metal ions and its influence on chromatin structure have yielded compelling results, revealing its nature as a zinc-dependent enzyme and a direct nucleosome reorganizer. These findings significantly expand our understanding of PARP2's multifaceted roles in cellular processes.

3.1. PARP2 Forms Stoichiometric Complexes with Nucleosomes Independent of Divalent Cations

Initial characterization of PARP2-nucleosome interactions revealed that PARP2 is capable of forming complexes with nucleosomes even in the absence of exogenous divalent cations. Electrophoretic Mobility Shift Assays (EMSAs) demonstrated that PARP2 begins to form complexes with nucleosomes at concentrations as low as 12.5 nM, with approximately 50% of free nucleosomes engaging in complex formation at around 40 ± 10 nM PARP2 (Fig. 1b).

As PARP2 concentration increased, multiple distinct bands with slower electrophoretic mobilities were observed in the gel, indicating the formation of complexes with varying stoichiometries (Fig. 1b). Up to five different complexes were discernible, suggesting that multiple PARP2 molecules can associate with a single nucleosome.



To precisely determine the stoichiometry, single-particle fluorescence intensity analysis of nucleosomes within these gel bands was performed. By measuring the Cy5 fluorescence intensity (ICy5) of individual particles, it was confirmed that the different complexes observed in the gel each contained a single nucleosome (Fig. 1c). This implies that the varying mobilities are indeed a result of different numbers of PARP2 molecules binding to one nucleosome (e.g., 1:1, 2:1, 3:1 PARP2:nucleosome ratios). This finding is consistent with PARP2's ability to form homodimers at higher concentrations (above 50-80 nM) [4], suggesting that dimerization of PARP2 molecules could contribute to the formation of higher-order complexes on the nucleosome. Furthermore, the relatively small size of PARP2 (approximately 60 kDa) suggests that several molecules could bind to a nucleosome without significant steric hindrance (Figure S7b).

Despite the formation of these multiple stoichiometric complexes, single-molecule FRET (smFRET) experiments revealed that PARP2 binding, in the absence of divalent cations, did not induce significant structural changes in the nucleosomal DNA. The EPR profiles of nucleosomes remained largely unchanged upon PARP2 addition across a wide range of concentrations (12.5-200 nM) (Fig. 1d). The profiles typically showed two subpopulations: a major peak at higher EPR values (around 0.8), corresponding to intact nucleosomes with closely positioned DNA gyres, and a minor peak at lower EPR values (around 0.03), likely representing histone-free DNA or nucleosomes undergoing transient "breathing" or unwrapping [46]. The preservation of these EPR profiles indicated that PARP2 binding alone, without specific metal ion modulation, does not significantly alter the overall conformation of nucleosomal DNA. This is a crucial baseline observation, distinguishing the role of PARP2 from other known chromatin remodelers that intrinsically alter nucleosome structure.

3.2. Zinc Ions Selectively Induce PARP2-Mediated Nucleosome Reorganization

The cellular nucleoplasm contains submillimolar concentrations of various divalent cations, including Ca2+, Mg2+, and Zn2+, which are known to influence chromatin structure and protein activity [47-50]. To investigate their impact, nucleosome-PARP2 interactions were examined in the presence of these ions.

Initially, the direct effect of these ions on nucleosome structure was assessed. Millimolar concentrations of Ca2+, Mg2+, or Zn2+ (e.g., 5 mM) were found to minimally affect the intrinsic structure of nucleosomes (Fig. 1e). This confirmed that any observed changes in nucleosome structure in the presence of PARP2 and these ions would be mediated by the protein, rather than direct ion-induced alterations to the nucleosome itself.

When PARP2 was incubated with nucleosomes in the presence of Ca2+ or Mg2+ ions (e.g., 5 mM), EMSA results showed the formation of multiple PARP2-nucleosome complexes with different stoichiometries, similar to those observed in the absence of divalent ions (Fig. 1f). Crucially, smFRET analysis of these complexes revealed no significant changes in the EPR profiles of the nucleosomes (Fig. 1g). This indicated that while Ca2+ and Mg2+ ions did not prevent PARP2 from binding to nucleosomes, they also did not facilitate any structural reorganization of the nucleosomal DNA by PARP2.

In stark contrast, the presence of Zn2+ ions (e.g., 0.3 mM) dramatically altered the nature of PARP2-nucleosome complexes (Fig. 1f). smFRET analysis showed a distinct shift in the EPR profile (Fig. 1g, h). A new, prominent peak emerged, centered at an EPR value of approximately 0.4. This new subpopulation corresponds to nucleosomes where the distance between the neighboring DNA gyres, at the sites of fluorescent labels, had significantly increased. This "gaping" or "unwrapping" of nucleosomal DNA was a direct consequence of PARP2 binding in the presence of Zn2+ ions.

Further titration experiments with varying Zn2+ concentrations revealed that this nucleosome reorganization effect was concentration-dependent, becoming apparent at Zn2+ concentrations above 75 μM and reaching saturation around 150 μM (Fig. 1h). Increasing Zn2+ concentrations up to 5 mM did not induce further changes, suggesting that the maximal structural alteration was achieved within this range. This indicates a specific, saturable binding event of Zn2+ that enables PARP2's remodeling activity.

To ascertain the extent of this Zn2+-mediated reorganization, nucleosomes with labels positioned at different regions of the DNA (Nm and Nd nucleosomes, Fig. 1a) were tested. Similar shifts in EPR profiles were observed for both Nm (labels at 35/112 bp) and Nd (labels at 57/135 bp) nucleosomes, with new peaks centered at EPR values of 0.4 and 0.5, respectively (Fig. 2b, c). This comprehensive change across different regions of the nucleosomal DNA suggests that the PARP2-induced structural rearrangement in the presence of Zn2+ affects the entire nucleosomal DNA, leading to a global increase in the distance between the DNA supercoils (Fig. 2d). This is distinct from simple unwinding from the ends, which would predict different FRET changes depending on label position.

A critical experiment involved the addition of EDTA, a strong chelator of divalent cations. When EDTA (10 mM) was added to PARP2-nucleosome complexes that had been pre-formed in the presence of Zn2+ (0.15 mM), the nucleosome structural changes were not reversed, even with a thirtyfold molar excess of EDTA over Zn2+ (Fig. 2e). This striking observation suggests that the Zn2+ ions involved in mediating the PARP2-induced nucleosome reorganization become sequestered or "hidden" within the complex, making them inaccessible to the chelator. This implies a tight, stable binding of Zn2+ within the PARP2-nucleosome complex, essential for maintaining the altered nucleosome conformation.

In summary, these results demonstrate a selective and profound effect of Zn2+ ions on PARP2's interaction with nucleosomes, leading to a reversible structural reorganization of the nucleosomal DNA. This effect is not observed with other physiologically relevant divalent cations like Ca2+ or Mg2+, highlighting a specific role for zinc in PARP2-mediated chromatin dynamics.

3.3. Zinc Ions Directly Bind to PARP2 and Induce Conformational Changes

Given the selective effect of Zn2+ on PARP2's ability to reorganize nucleosomes, the next logical step was to investigate whether Zn2+ ions directly interact with PARP2 and induce conformational changes in the enzyme itself. While no canonical zinc-finger domains were initially predicted for PARP2 based on primary sequence analysis, this does not preclude the formation of zinc-binding sites within the folded protein.

Circular Dichroism (CD) spectroscopy was employed to probe the secondary structure of full-length PARP2. The CD spectra of PARP2 in the absence of divalent cations or in the presence of Ca2+ or Mg2+ ions were largely similar (Fig. 2f and S2b). However, a distinct difference was observed in the CD spectrum recorded in the presence of Zn2+ ions (e.g., 0.3 mM). This spectral difference indicated that Zn2+ ions induce subtle yet significant conformational changes in the overall secondary structure of PARP2 (Figure S2a).

Direct interaction of Zn2+ ions with PARP2 was further confirmed using intrinsic tryptophan fluorescence spectroscopy. PARP2 contains tryptophan residues, whose fluorescence is highly sensitive to their local microenvironment. Upon the addition of Zn2+ ions, the intensity of tryptophan fluorescence of PARP2 was observed to increase (Fig. 2g). This change in fluorescence intensity, without a significant shift in the emission maximum, is indicative of alterations in the microenvironment surrounding one or more tryptophan residues, consistent with direct binding of Zn2+ and a subsequent conformational rearrangement of the protein. Since Zn2+ alone does not affect the structure of intact nucleosomes (Fig. 1e), these Zn2+-induced conformational changes in PARP2 are highly likely to be the driving force behind the altered mode of PARP2 interaction with nucleosomes and the subsequent

reorganization of nucleosome structure within the complex.

3.4. The WGR Domain of PARP2 Contains Zinc-Binding Sites and Mediates Zinc-Dependent Nucleosome Reorganization

To pinpoint the region within PARP2 responsible for Zn2+ binding and the subsequent nucleosome reorganization, a detailed analysis of PARP2's domains was undertaken. While initial bioinformatics analysis using tools like ZincBind Predict [54] did not reveal canonical zinc-binding sites (ZnBSs) from the primary sequence, this is often the case for sites formed by tertiary structure. Zinc ions are typically coordinated by histidine, cysteine, glutamate, and aspartate residues [55].

Structural analysis of known PARP2 domains (e.g., WGR domain, PDB entry: 6F5B; catalytic and α-helical domains, PDB entry 4ZZY) revealed two potential zinc-binding sites exclusively localized within the WGR domain (Fig. 3a). Site 1 was identified as being formed by residues E97, C98, and H160, while Site 2 was formed by residues H106, C109, and E138. Although these residues were not in close proximity in the crystal structure of the WGR domain obtained without zinc [25], guided molecular dynamics (MD) simulations were performed to model the WGR domain in the presence of two Zn2+ ions. The MD simulations demonstrated that both the free WGR domain and the WGR-Zn2+ complex maintained structural stability and size over a 400 ns simulation period (Figure S3a, S3b), suggesting that these proposed Zn2+ binding sites could indeed form stable coordination environments within the folded protein. These predicted ZnBSs could be classified as belonging to the CIEIH1 family according to the ZincBind database [56], a family known to include sites with high affinity for Zn2+ [60, 61].

To experimentally validate these predictions, the recombinant WGR domain (residues 77-220) was purified, and its interaction with Zn2+ ions was studied. CD spectroscopy of the isolated WGR domain showed significant structural changes upon the addition of Zn2+ ions (e.g., 0.5 mM), with notable alterations in its secondary structure content (Fig. 3b, c). In contrast, Ca2+ or Mg2+ ions induced only minimal structural changes in the WGR domain (Figure S2c), mirroring the observations with full-length PARP2.

Tryptophan fluorescence spectroscopy of the WGR domain further confirmed direct Zn2+ binding. The WGR domain contains three tryptophan residues (Trp 148, 151, and 188). Upon Zn2+ addition, the intensity of tryptophan fluorescence of the WGR domain increased considerably, without changes in the spectrum shape or maximum (Fig. 3d). This effect was similar to that observed for full-length PARP2 (Fig. 2g), strongly supporting the hypothesis that Zn2+ interacts directly with the WGR domain, leading to changes in the microenvironment of its tryptophan residues. The observed changes in fluorescence occurred at Zn2+ concentrations between 0.4 and 5 μM, and the calculated apparent dissociation constant (Kd) for the WGR-Zn2+ complex was approximately 2.0 ± 0.4 μM (Figure S4). This value, while seemingly moderate, could be an overestimate due to residual EDTA in the reaction solution competing for Zn2+ binding. Molecular modeling further supported these findings, predicting that Zn2+ binding to the WGR domain would lead to increased solvent accessibility for at least one tryptophan residue (Trp 148) (Fig. 3e). While Ca2+ and Mg2+ ions also bound to the WGR domain, their affinities were lower (Kd values of 29 ± 14 μM for Ca2+ and 180 ± 80 μM for Mg2+), and their binding did not induce the same extent of structural reorganization as Zn2+ (Figure S5).

Crucially, the ability of the WGR domain to bind to nucleosomes and induce structural changes in a Zn2+-dependent manner was investigated. EMSA showed that the WGR domain binds to nucleosomes both in the presence and absence of Zn2+ ions (Fig. 3f). However, smFRET microscopy demonstrated that while WGR binding alone (without Zn2+) did not significantly alter nucleosome structure (Fig. 3g), the presence of Zn2+ ions (e.g., 2 mM) resulted in a considerable change in the nucleosome structure within the WGR-nucleosome complex (Fig. 3h). A new peak, similar to that observed with full-length PARP2, appeared in the EPR profile with a maximum at 0.4, indicating an increase in the distance between nucleosomal DNA gyres. The striking similarity between the Zn2+-dependent EPR profile changes induced by full-length PARP2 and by the isolated WGR domain (compare Fig. 2a and Fig. 3h) strongly suggests that the WGR domain is the primary mediator of the Zn2+-dependent nucleosome reorganization activity of PARP2.

These results conclusively establish that the WGR domain of PARP2 contains functional zinc-binding sites. The binding of Zn2+ to these sites induces conformational changes within the WGR domain, which in turn enables PARP2 to reorganize nucleosome structure.

3.5. Differential Modulation of PARP2 Catalytic Activity by Magnesium and Zinc Ions

Beyond its structural role in nucleosome reorganization, the catalytic activity of PARP2, specifically its autoPARylation, was also found to be differentially modulated by divalent cations.

Upon addition of NAD+ substrate to PARP2-nucleosome complexes, PARP2's catalytic function is activated, leading to its autoPARylation and the PARylation of other proteins, including histones (Fig. 4a). This PARylation process is concentration-dependent on NAD+ (Fig. 4a). A key observation was that autoPARylation of PARP2 leads to its dissociation from the nucleosome complexes, both in the presence and absence of Zn2+ ions (Fig. 4b, c). This dissociation is likely driven by the accumulation of negative charges on PARylated PARP2, leading to

electrostatic repulsion from the negatively charged nucleosomal DNA [24, 57, 58]. Importantly, the nucleosome's intact conformation is restored after PARP2 dissociation (Fig. 4b, c), indicating that the Zn2+-dependent structural changes induced by PARP2 are fully reversible.

The efficiency of PARP2-mediated PARylation was found to be significantly affected by the presence of Mg2+ and Zn2+ ions, but in opposing ways (Fig. 4a). Mg2+ ions were observed to increase the efficiency of PARylation, suggesting a stimulatory role. In contrast, Zn2+ ions consistently decreased the efficiency of PARylation, indicating an inhibitory effect.

A particularly intriguing finding emerged from experiments involving EDTA. When EDTA was added in excess to reactions containing Mg2+, it completely reversed the stimulatory effect of Mg2+ on PARylation (Fig. 4a). This is consistent with Mg2+ binding to an exposed site on PARP2 that is accessible to the chelator. However, when EDTA was added to reactions containing Zn2+, it not only abolished the Zn2+-induced suppression of PARylation but surprisingly led to a strong enhancement of PARylation efficiency (Fig. 4a). This complex response to EDTA, especially when contrasted with the non-reversal of nucleosome structural changes by EDTA (Fig. 2e), suggests the existence of at least two functionally distinct zinc-binding sites on PARP2.

One Zn2+ binding site appears to be exposed to the solution and, when occupied by Zn2+, negatively regulates PARP2 catalytic activity. This site is accessible to EDTA, and its chelation by EDTA relieves the inhibition, leading to enhanced PARylation. The second Zn2+ binding site, likely located within the WGR domain at the interface of PARP2-nucleosome interaction, becomes hidden or protected within the complex, rendering it inaccessible to EDTA. This second site is responsible for mediating the Zn2+-dependent nucleosome reorganization. Furthermore, the data suggest that the occupancy of this "hidden" site may be linked to the observed enhancement of catalytic activity when Zn2+ is removed from the first, exposed site by EDTA. This is consistent with previous observations that the WGR domain contributes significantly to the DNA-dependent catalytic activity of PARP2 [62]. Importantly, the Zn2+ ions primarily affect the autoPARylation activity, rather than significantly altering PARP2's affinity for the nucleosome itself.

These results highlight a sophisticated regulatory mechanism for PARP2, where different divalent cations, and even different binding sites for the same cation (Zn2+), can fine-tune its enzymatic activity and its ability to remodel chromatin.

4. DISCUSSION

The findings presented herein fundamentally reshape our understanding of Poly(ADP-ribose) Polymerase 2, revealing its unexpected nature as a zinc-dependent enzyme and a direct nucleosome reorganizer. These discoveries underscore the intricate regulatory mechanisms governing PARP2's diverse cellular functions and carry significant implications for DNA damage response, chromatin dynamics, and the development of targeted therapeutics.

4.1. PARP2: A Zinc-Dependent Metalloenzyme

The classification of PARP2 as a zinc-dependent enzyme is a pivotal insight. While many proteins, particularly those involved in nucleic acid binding, utilize zinc fingers for structural stability and specific recognition [51, 55], the explicit identification of functional zinc-binding sites within PARP2, especially within its WGR domain, was not previously a prominent feature of its characterization. Our data, supported by both experimental evidence (CD and fluorescence spectroscopy) and computational modeling (MD simulations and bioinformatics predictions), clearly demonstrate that Zn2+ ions directly interact with PARP2, inducing subtle but significant conformational changes [25, 26, 35, 54, 55, 56, 60].

The observed Kd for Zn2+ binding to the WGR domain (approximately 2 μM) suggests a physiologically relevant affinity. Cellular zinc concentrations, while dynamic, typically range from submicromolar to low millimolar, with a significant fraction localized within the nucleus [65, 66]. Nuclear Zn2+ concentrations are known to fluctuate during the cell cycle and in response to various stresses, such as nitrosative stress [67, 68, 69, 70, 71]. This dynamic availability of Zn2+ suggests that it could serve as a crucial regulatory signal, fine-tuning PARP2's activity and its mode of interaction with chromatin. The sensitivity of PARP2 to zinc levels implies that disruptions in cellular zinc homeostasis, which are associated with various pathologies [74], could directly impact DNA repair efficiency and other PARP2-mediated processes. This adds a novel layer of complexity to PARP2 regulation, distinct from its well-established activation by DNA breaks.

The existence of two functionally distinct zinc-binding sites within PARP2, as suggested by the differential effects of EDTA on its catalytic activity and nucleosome remodeling, is particularly intriguing. One site, exposed to the solvent and accessible to chelators, appears to exert a negative regulatory effect on PARP2's autoPARylation activity. The other, likely embedded within the WGR domain and becoming "hidden" upon nucleosome complex formation, mediates the structural reorganization of nucleosomes. This dual regulatory mechanism allows for a sophisticated control system where Zn2+ can simultaneously influence both the catalytic output and the physical interaction of PARP2 with its chromatin substrate. This also provides a plausible explanation for how the WGR domain, known to contribute to PARP2's DNA-dependent catalytic activity [62], might do so in a zinc-dependent manner.

4.2. PARP2 as a Direct Nucleosome Reorganizer: Beyond

PARylation

Our findings establish PARP2 as a direct nucleosome reorganizer, capable of inducing reversible structural changes in nucleosomal DNA, specifically an increase in the distance between DNA gyres [28, 32, 34, 46, 63]. This activity is critically dependent on Zn2+ ions and is mediated by the WGR domain. This represents a significant advancement in understanding PARP2's role in chromatin dynamics, moving beyond its established function as a DNA damage sensor that recruits other factors.

The ability of PARP2 to directly unwrap or "gape" nucleosomal DNA, even in the absence of extensive PARylation, positions it as an active participant in the initial stages of chromatin decompaction at sites of DNA damage. While PARP1 is well-known for its robust PARylation activity, which leads to charge-driven chromatin relaxation and recruitment of repair proteins [72], PARP2's direct remodeling capability suggests a complementary or potentially redundant mechanism. This intrinsic ability to alter nucleosome structure might be particularly important for specific types of DNA lesions, or in contexts where PARP1 activity is limited or absent. The reversibility of these nucleosome structural changes upon PARP2 dissociation (following autoPARylation) is also critical, ensuring that chromatin structure can be restored once repair is complete.

The precise molecular mechanism by which PARP2, via its WGR domain and in a Zn2+-dependent manner, induces this nucleosome reorganization warrants further investigation. It is unlikely to be simple DNA unwinding from the ends or nucleosome sliding, as these mechanisms would predict different FRET changes than those observed across multiple labeling positions [63]. Instead, the data support a model where PARP2 binding, facilitated by Zn2+, induces a global increase in the distance between the DNA supercoils along the entire nucleosome (Fig. 2d, 5). This "gaping" could be achieved through conformational changes in the WGR domain that exert mechanical force on the nucleosomal DNA, or by altering the interaction interface between DNA and histones.

4.3. Interplay of Cations in Regulating PARP2 Function

The differential effects of Mg2+ and Zn2+ ions on PARP2's catalytic activity and nucleosome remodeling highlight a sophisticated regulatory network (Fig. 5). Mg2+ ions generally enhance PARylation, while Zn2+ ions, depending on their binding site, can either inhibit or, surprisingly, enhance it upon chelation of the exposed site. This suggests that the balance of these ubiquitous divalent cations within the cell could serve as a dynamic rheostat for PARP2 function.

Transient changes in the concentration of these cations, which are known to occur in response to various cellular signals and stresses [47, 48, 68, 69, 70, 71], could differentially modulate PARP2's catalytic output and its ability to remodel chromatin. For instance, a localized increase in nuclear Zn2+ could prime PARP2 to reorganize nucleosomes, making DNA lesions more accessible, while simultaneously modulating its PARylation activity to control the timing of repair protein recruitment and PARP2 dissociation. This dynamic interplay ensures that the cell can rapidly adapt its DNA damage response based on the prevailing ionic environment.

The observation that PARP1 autoPARylation is also influenced by divalent cations [73] further emphasizes a broader role for these ions in regulating the entire PARP family and the DDR. Synergistic effects of cation combinations on PARP1 activity have been reported [73], suggesting a complex regulatory landscape where different combinations of Zn2+, Mg2+, and Ca2+ could fine-tune the dynamics, structure, and length of PAR polymers, as well as the extent of PARylation of nuclear proteins. Given that deficiencies in magnesium and zinc are linked to various pathologies [74, 75, 76], it is plausible that ion-dependent modulation of PARP1 and PARP2 activity contributes to the development or progression of these diseases.

4.4. Therapeutic Implications and Future Directions

The elucidation of PARP2 as a zinc-dependent enzyme and a direct nucleosome reorganizer carries significant implications for the design and application of PARP inhibitors in cancer therapy. Current PARP inhibitors, while effective, often exhibit off-target toxicities due to their broad-spectrum inhibition of both PARP1 and PARP2 [12, 13, 14, 15]. The finding that inactive PARP2 can independently contribute to severe anemia [16] underscores the critical need for more selective inhibitors.

Understanding the unique zinc-binding sites and their functional roles in PARP2 opens new avenues for rational drug design. For instance, compounds that specifically target the zinc-binding site(s) of PARP2, or that modulate Zn2+ availability in the nucleus, could potentially offer a novel approach to selectively inhibiting PARP2 or altering its chromatin remodeling activity without affecting PARP1. Such selective modulation could lead to PARP inhibitors with improved safety profiles and reduced hematological toxicities. Furthermore, combination therapies that exploit the distinct mechanisms of PARP1 and PARP2, perhaps by combining a PARP1-selective inhibitor with a PARP2 modulator that targets its zinc-dependent functions, could enhance therapeutic efficacy while minimizing adverse effects.

Future research should focus on several key areas:

- High-Resolution Structural Studies: Obtaining high-resolution structures of full-length PARP2 in complex with nucleosomes and Zn2+ ions (e.g., via Cryo-EM) is paramount. This would precisely map the interaction interfaces, the Zn2+ coordination environment, and the conformational changes induced in both PARP2 and the nucleosome.

- Molecular Mechanism of Remodeling: Detailed biochemical and biophysical studies are needed to fully unravel the molecular mechanism by which the WGR domain, upon Zn2+ binding, induces nucleosome gaping. This could involve force microscopy or advanced computational simulations to visualize the dynamic process.

- In Vivo Validation: Translating these in vitro findings to cellular and in vivo contexts is crucial. This would involve using zinc sensors [71], genetic manipulation of PARP2's zinc-binding sites, and cellular assays to confirm the physiological relevance of Zn2+ regulation of PARP2 and its impact on chromatin accessibility and DNA repair in living cells and organisms.

- Role in Other Processes: Given PARP2's involvement in spermatogenesis, adipogenesis, and T-cell development [19, 20, 21, 22], future studies should explore whether its zinc-dependent nucleosome remodeling activity plays a role in these non-DNA repair functions.

- Therapeutic Development: The development of novel small molecules that selectively target PARP2's zinc-binding sites or modulate zinc homeostasis to impact PARP2 activity could lead to a new generation of more precise and less toxic PARP inhibitors.

5. CONCLUSION

In conclusion, the emerging evidence presented in this article unequivocally establishes Poly(ADP-ribose) Polymerase 2 as a zinc-dependent enzyme and a direct nucleosome reorganizer. This dual functionality, intricately regulated by the dynamic interplay of divalent cations, particularly Zn2+ and Mg2+, adds significant depth to our understanding of PARP2's fundamental biology. The WGR domain has been identified as the key mediator of Zn2+ binding and subsequent nucleosome remodeling. These novel insights illuminate how PARP2 actively participates in shaping chromatin architecture to facilitate DNA repair and potentially other essential nuclear processes. The profound implications of these discoveries extend to the therapeutic realm, offering promising avenues for the rational design of more selective PARP inhibitors with improved efficacy and reduced adverse effects, ultimately advancing cancer therapy and our understanding of human health and disease.

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