Toward a Quantitative Analysis of PARP-1 and Poly(ADP-ribosyl)ation in Cellular Senescence

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ABSTRACT

Aging is a complicated and multifactorial phenomenon. Model systems involving the induction of replicative senescence in cultured cells have been indispensable in elucidating some of the mechanisms underlying this complex process. An understanding of how and why cellular senescence occurs is thus critical to the field of aging research. While there is much correlative evidence to suggest a connection between poly(ADP-ribose) (PAR) and mammalian longevity, no studies have been done to explore a possible role for PARP-1 — the enzyme responsible for synthesis of 90% of cellular PAR — in mechanisms of senescence. Furthermore, many techniques currently used for analysis of protein poly(ADP-ribosylation) are fraught with imprecision. We therefore sought to address these issues both by developing methods for the unambiguous analysis of poly(ADP-ribosylation) by mass spectrometry, and by exploring the role of PARP-1 in nicotinamide-mediated cellular lifespan extension.

Due to the challenges introduced by PAR's biochemical characteristics, successful mass spectrometric analysis of poly(ADP-ribosylation) will require the use of techniques to reduce the mass, charge, and heterogeneity of the polymer, as well as methods to enrich for poly(ADP-ribosyl)ated protein. To this end, we evaluated the effectiveness of several approaches, including ammonium sulfate fractionation, boronate affinity chromatography, snake venom phosphodiesterase digestion, manipulation of PARP-1 reaction conditions, and immobilized metal affinity chromatography (IMAC) for the preparation of poly(ADP-ribosyl)ated protein samples prior to MS analysis using both MALDI-TOF and Q-TRAP LC-MS. Based on this work, we developed a three-tiered scheme that may provide the first ever identification of poly(ADP-ribosyl)ated peptides from full-length wild-type PARP-1 by mass spectrometry.
Past work in our laboratory has demonstrated that nicotinamide (NAM), a component of vitamin B₃, significantly extends the replicative lifespan of human fibroblasts. In order to help elucidate the role of PARP-1 in cellular senescence, we then analyzed the poly(ADP-ribosyl)ation response of aging cells undergoing NAM-mediated lifespan extension. While NAM is a known PARP-1 inhibitor, we found that oxidative stress-induced poly(ADP-ribosyl)ation is increased, not decreased, in NAM-treated cells. We propose that supplemented NAM is taken up by the NAD salvage pathway, ultimately leading to increased cellular NAD and extending replicative lifespan by both preventing PARP-mediated NAD depletion and upregulating SIRT1. We further propose that the demonstrated protective effects of NAM treatment in a number of disease models are due not to PARP-1 inhibition as is commonly assumed, but to upregulation of NAD salvage.

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List of Abbreviations

3-AB: 3-aminobenzamide
AIF: apoptosis-inducing factor
ATCC: American Type Culture Collection
BER: base excision repair
bio-NAD: biotinylated NAD
BSA: bovine serum albumin
CDK: cyclin-dependent kinase
DAB: 3,3’-diaminobenzidine
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: dimethyl sulfoxide
DNA-PK: DNA-dependent protein kinase
DSB: double-strand break
EDTA: ethylenediaminetetraacetate
ELISA: enzyme-linked immunosorbent assay
FBS: fetal bovine serum
HR: homologous recombination
HRP: horseradish peroxidase
IMAC: immobilized metal ion affinity chromatography
IPTG: isopropyl β-D-1-thiogalactopyranoside
LB: Luria-Bertani
mAB: monoclonal antibody
MORFS: microscopic optical replicas for fluorescence assays
MS: mass spectrometry, mass spectrometric
MWCO: molecular weight cut-off
NAD⁺: nicotinamide adenine dinucleotide
NAM: nicotinamide
NAMPT: nicotinamide phosphoribosyl transferase
NHEJ: non-homologous end-joining
NMAT: nicotinamide mononucleotide adenylyl transferase
NMN: nicotinamide mononucleotide
pADPr: poly(ADP-ribose)
PAR: poly(ADP-ribose)
PARP: poly(ADP-ribose) polymerase
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PD: population doubling

PMSF: phenylmethanesulphonylfluoride

PTM: post-translational modification

ROS: reactive oxygen species

SA-β-gal: senescence-associated β-galactosidase

SAHF: senescence-associated heterochromatin foci

SSBR: single-strand break repair

SV-PDE: snake venom phosphodiesterase

TBS-T: Tris buffered saline – Tween 20

TCA: Trichloroacetic acid

TFA: Trifluoroacetic acid
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Literature Review and Research Aims

Introduction

The poly(ADP-ribose) polymerases (PARPs) comprise a family of eukaryotic proteins that catalyze the formation of long chains of ADP-ribose units resulting from cleavage of donor NAD$^+$ molecules, with subsequent release of nicotinamide [1–3]. Their existence was first postulated in the 1960’s when a nicotinamide mononucleotide-dependent phosphate-incorporating activity was discovered in a preparation of chicken liver nuclei [4]. The nature of the resulting polymer, which may either exist as a free macromolecule or associate with protein — including the PARPs themselves — via covalent modification or noncovalent interaction, was eventually determined by hydrolysis with snake venom phosphodiesterase (SV-PDE) followed by analysis of the digestion products [2, 5]. Further details of poly(ADP-ribose) (PAR) structure have since been revealed (Figure 1).

Poly(ADP-ribose)ylation regulates protein function by altering enzymatic activities and/or macromolecular interactions [19]. Each unit of the PAR polymer contains an adenine moiety capable of base stacking and hydrogen bonding, as well as two phosphate groups which contribute considerable negative charge. The biochemical consequences of its covalent attachment to or noncovalent association with a protein are thus potentially profound. On one hand, PAR participates in repulsive interactions with other biomolecules due to steric hindrance and/or electrostatic repulsion [20–22], a property which may be responsible for its suppressive effect on the activity of enzymes such as topoisomerases [23, 24], DNA polymerases and ligases.
Snake venom phosphodiesterase (SV-PDE) hydrolysis products | Branch point: 1 → 2 linked

Repeat unit: 1' → 2'-linked

Chain lengths of up to 200 residues have been reported, with the repeat unit having both \(\alpha\)-(1-2)-ribofurananosidic and pyrophosphate linkages [11–15]. The polymer contains branch points approximately every 20-50 units [16], again linked via an \(\alpha\)-(1-2)ribofurananosidic bond, although in this case the aglycone ribofuranose does not contain an adenine moiety [17, 18].

(B) The overall PAR polymer structure was elucidated by selective degradation. SV-PDE cleavage products are shown along with the "reducing end" moiety (ribose-5-phosphate), which remains attached to the protein.
[25, 26], RNA polymerases [27, 28], and the PARPs themselves [29]. Conversely, certain nuclear proteins, including histones and p53, form strong attractive noncovalent interactions with the polymer [30–33]. These interactions may be quite specific; analysis of several PAR-binding DNA repair proteins revealed a semi-conserved 20 amino-acid PAR binding motif [32], and Karras et al. recently discovered that the macro domain, a 190-residue sequence found in proteins from diverse biological pathways, is a high-affinity PAR-binding module [34]. The strength of the protein/polymer association varies with the type of protein and the polymer chain length, with longer chains generally binding more tightly than shorter oligomers [33]. It has been suggested that covalent poly(ADP-ribosylation) serves mostly to directly alter enzymatic activity, whereas noncovalent association is a mechanism for the recruitment of target proteins to specific subcellular locations. A protein may participate in both types of interactions; for example, it may be targeted to a particular site via noncovalent binding, and then have its enzymatic activity modified by covalent poly(ADP-ribosylation) [19].

In unstimulated cells, PAR levels are typically quite low and the majority of cellular ADPr exists as monomers or oligomers of 2 to 11 units in length [16]. However, PARP activity increases up to 10- to 500-fold in the presence of DNA damage [35, 36]; the resulting PAR chains may be up to 200 residues long and their formation is accompanied by a measurable reduction in cellular NAD$^+$ as the cofactor is cleaved and consumed [37, 38]. It has been shown that average PAR chain length and degree of branching are affected by cellular growth conditions. For example, cells treated with the DNA alkylating agent MNNG produce polymers of up to 67 residues with two branch points, whereas cells treated with hyperthermia prior to DNA damage produce polymers of up to 244 residues with 6 branch points [39]. While neither the mechanism behind these differences nor their effect on cellular physiology is well understood, the heterogeneity of cellular PAR suggests the existence of a “poly(ADP-ribose) code” which determines outcome of various signaling pathways [40].

In vivo, PAR levels are tightly regulated by the opposing actions of PARPs and poly(ADP-ribose) glycohydrolase (PARG), which hydrolyzes the glycosidic linkages between ADP-ribose
units and releases free ADP-ribose [41] for subsequent degradation to AMP and ribose-5-phosphate by ADPr pyrophosphatases [42, 43]. PARG has differential affinity for various types of PAR; both in vitro and in vivo experiments have demonstrated that long, linear polymer chains of the sort synthesized after DNA damage are degraded more quickly than the short, branched chains that predominate in unstimulated cells. In fact, the major fraction of PAR in cells treated with DNA-damaging agents has a half-life of less than one minute, compared to a half life of over 7 hours for constitutively synthesized polymer [44, 45]. This suggests that while PARP participates in DNA damage signaling, it has housekeeping functions as well. Disruption of the PARG gene in mice results in increased sensitivity to PARP-1-dependent cytotoxicity and early embryonic lethality accompanied by PAR accumulation, illustrating the importance of PAR metabolism to cellular and organismal survival [46].

While sequence homology searches have identified seventeen different putative PARPs in the human proteome, only six of these have thus far been shown to have poly(ADP-ribosyl)ation activity [47]. The most abundant and enzymatically active of the PARPs, PARP-1, is responsible for approximately 90% of cellular PAR and is the main acceptor of the polymer in both isolated nuclei and intact permeabilized cells [48, 49]. It is also by far the most-studied member of the protein family. Despite this fact, a number of issues have clouded the elucidation of its cellular functions. Perhaps most problematically, the protein's enzymatic activity has repeatedly been described in the literature – in direct contradiction of early studies demonstrating histone-mediated PARP activation [50] – as having complete dependence upon the presence of DNA strand breaks [16, 51, 52]. As a result, PARP-1's involvement in the DNA damage response is well-documented (reviewed in [53]), whereas its possible roles in other cellular processes have remained comparatively unexplored. Only in recent years has it become more widely acknowledged that PARP-1 activity is stimulated by a much wider range of stimuli than previously thought – including certain hairpin and loop DNA structures, nucleosomes, and various protein binding partners [54–58] – in addition DNA damage caused by agents such as gamma radiation, topoisomerase inhibitors, methylating compounds, and reactive oxygen species. It has become
apparent that PARP-1’s influence extends far beyond the DNA damage repair arena; the enzyme is now implicated in an ever-expanding list of diverse physiological processes and pathologies ranging from chromatin remodeling to inflammation [59, 60].

**PARP-1 Structural Features**

PARP-1’s structure-function relationship has been extensively studied. In the early 1980’s a series of proteolysis-based experiments using radiolabeled NAD$^+$ identified three distinct domains (Figure 2), including a catalytic domain, DNA binding domain, and an “automodification” domain which was thought to contain from fifteen [61] to twenty-eight [62] sites of covalent poly(ADP-ribosyl)ation. While the extent and nature of these modifications is controversial [47], it is generally believed that covalent poly(ADP-ribosyl)ation in the automodification region results in inactivation of the enzyme due to electrostatic repulsion between PAR and DNA [29]. This interpretation, however, relies upon the questionable assumption that PARP-1 activity is absolutely DNA-dependent; considering recent evidence to the contrary, it is likely that there are other factors at play in automodification-mediated PARP-1 inactivation.

![Figure 2. PARP-1 structural features.](image)

**Figure 2.** PARP-1 is a 113 kDa protein (1014 amino acids) with several functional domains [63–65]. The DNA-binding domain located at the N-terminus contains two zinc fingers responsible for protein-DNA interaction, as well as a bipartite nuclear localization signal [66] and a caspase cleavage site (not shown). The automodification domain between residues 383 and 524 [11, 62] contains a BRCT domain, which is found predominantly in proteins responsive to DNA damage and is the main site of interaction between PARP-1 and its various protein binding partners. The catalytic domain is the smallest PARP-1 fragment with catalytic activity and resides at the C-terminus [67]. The active site contains a 50-amino acid "PARP signature" motif which has 100% conservation among vertebrates [55].

Human and bovine PARP-1 cDNAs were isolated in 1987 and 1990, respectively, and have since been used as probes for the isolation of PARP-1 cDNA from rat, mouse, chicken, *Xeno-
pus, Drosophila, Sarcophaga peregrina, Arabidopsis, and maize. The protein sequence (Figure 3) is highly conserved among species; human PARP-1 demonstrates 92 - 98% homology with the mouse, rat, and bovine enzymes, and most amino acid changes found between sequences are conservative. Moreover, regions of high sequence similarity correspond neatly to the experimentally-determined functional domains of the protein, indicating that the enzyme structure has been conserved during evolution. The catalytic domain in particular contains a 50 amino acid domain that is 100% conserved among vertebrates and is thus known as the “PARP signature sequence” [68]. This region contains the substrate-binding site as well as residues critical to PAR chain initiation, elongation, and branching [40].

Figure 3. PARP-1 amino acid sequence and post-translational modifications. PARP-1’s protein sequence is highly conserved between species. Phosphorylated and acetylated residues have been verified in human cell lines by mass spectrometry [69, 70]. Poly(ADP-ribosyl)ation of multiple glutamate residues in the automodification domain has been suggested but not experimentally proven, and should still be considered putative [47].
Known Cellular Functions of PARP-1

Though the field of poly(ADP-ribosyl)ation is rapidly expanding and new roles for PARP-1 and PAR are continually being discovered, the enzyme's involvement in several major cellular processes is relatively well-established. The function of PARP-1 in DNA damage repair, modulation of cell death, and epigenetic control is discussed below.

PARP-1 and DNA Damage Repair. PARP-1 was originally purified from chicken liver nuclei and eventually found to demonstrate exclusively nuclear localization. This, in conjunction with the discovery that various chromatin components appeared to be ADP-ribosylated [71, 72], led early researchers to postulate a role for PARP-1 in chromatin maintenance. This idea was given credence when it was found that chromatin-associated PARP activity was highest in unfolded, transcriptionally active regions (euchromatin), whereas tightly-folded, transcriptionally inert regions (heterochromatin) contained little ADP-ribosylation activity [73]. Subsequent experiments confirmed PARP's effect on chromatin structure: upon poly(ADP-ribosyl)ation, purified nucleosomes adopt an open, relaxed conformation verifiable through both electron microscopy and sedimentation velocity studies [74]. The appearance of this relaxed DNA is similar to that of histone H1-depleted chromatin, suggesting that poly(ADP-ribosyl)ation alters DNA-histone interactions by reducing histone's affinity for DNA [7]. As DNA repair requires that chromatin be accessible to the repair machinery, these findings implied a role for PARP-1 in the process. This hypothesis would also explain the observation that treatment with DNA-damaging agents — specifically, those resulting in lesions that mainly activate the base excision repair (BER) and/or single-strand break repair (SSBR) pathways — causes a dramatic increase in both cellular PARP-1 activity and PAR levels [75–77], with a concurrent decrease in NAD⁺ [78–80].

Though experiments involving chemical inhibition [81, 82], expression of dominant negative mutants [83–85], and introduction of antisense oligonucleotides [86] suggested that PARP-1 was necessary for efficient base excision repair, the development of PARP-1 knockout mice in 1995 was critical in the verification of this connection. Embryonic cell lines derived
from these animals, as well as the animals themselves, were found to be severely deficient in DNA repair, featuring abnormal sensitivity to alkylating agents and ionizing radiation [87–91]. Specifically, absence of PARP-1 resulted in significantly delayed repair of DNA lesions by the BER pathway [92], indicating that while PARP-1 is not absolutely necessary for BER function, it does speed the process significantly.

Subsequent work using immunoprecipitation and a yeast two-hybrid system identified PARP-1 as part of a complex including XRCC1, DNA ligase III, and DNA polymerase β [93]; these findings were later verified in mammalian cells [94]. Deletion experiments indicated that the BRCT domain of XRCC1 interacts with PARP-1’s DNA-binding domain and automodification region, which also contains a BRCT domain. PARP-1 in turn interacts with the N-terminal regions of DNA ligase III and DNA polymerase β. As XRCC1 has also been shown to associate with the BRCT motifs of these repair proteins [95, 96], it has been concluded that the BER machinery consists of a multiprotein complex including DNA damage sensors (PARP-1 and DNA ligase III), a scaffold protein (XRCC1), and DNA repair proteins (DNA polymerase β and DNA ligase III), and that the integrity of the complex is maintained by interactions between the BRCT motifs of its various components [93]. PARP-1 is required for recruitment of XRCC1 to the complex [97]; both elimination of PARP-1 and chemical inhibition of PAR synthesis reduce or abolish XRCC1 targeting to sites of DNA damage [67, 94], which in turn impedes DNA damage repair as XRCC1 is an activator of BER/SSBR enzymes [98]. This likely explains the severe deficiencies in BER exhibited by PARP-1 knockout cells and animals.

PARP-1 has also been shown to play a role, however less critical, in DNA double strand break (DSB) repair. DSBs, which are commonly caused by ionizing radiation, nucleases, or replication fork collapse, are serious lesions with consequences ranging from large- and small-scale deletions to chromosome loss to cell death [99]. Over time they contribute to genomic instability, a hallmark of cancer cells and one of the driving forces of aging in eukaryotes [100]. DSB repair occurs via either homologous recombination (HR), in which large homologous regions elsewhere in the genome are used as templates for DNA synthesis, or non-homologous
end-joining (NHEJ), a less-accurate repair in which templates are provided by short homologous DNA sequences known as “microhomologies” [99]. NHEJ mainly occurs via the “classical” pathway, in which Ku binds to DNA double strand breaks, complexes with the DNA-dependent protein kinase catalytic subunit to create the full DNA-dependent protein kinase, and serves as a scaffold for the targeted assembly of other proteins involved in NHEJ [101]. When the classical pathway is compromised, however, NHEJ may proceed via an alternative PARP-1-dependent pathway employing the PARP-1/DNA ligase III/XRCC1 complex from the SSBR machinery. Pathway selection appears to be driven by competition for double strand breaks between Ku and PARP-1; as the affinity of Ku for DNA ends is higher than that of PARP-1, the alternative pathway (which is slower and more error-prone) is used only in the event that the classical pathway malfunctions [102].

Mediation of cell death by PARP-1. It has long been known that cells treated with high concentrations of DNA-damaging agents exhibit a sharp decrease in glycolytic activity followed by cell death. The discovery that cellular NAD$^+$ is significantly reduced under such conditions — and that this reduction is abrogated by concurrent treatment with PARP-1 inhibitors — led to what is now known as the “PARP suicide hypothesis” [40]. According to this model, massive DNA damage causes PARP-1 overactivation and subsequent NAD$^+$ depletion [103], which hampers NAD$^+$-dependent ATP-producing metabolic pathways such as mitochondrial respiration and glycolysis. To make matters worse, NAD$^+$ synthesis is then ramped up by the ATP-dependent enzymes phosphoribosyl transferase and nicotinamide mononucleotide adenyllyl transferase in an effort to restore cellular pools of the cofactor [104]. The end result is depletion of cellular ATP resulting in rapid necrosis [105]. (In contrast to the programmed form of cell death known as apoptosis, necrosis culminates in plasma membrane breakdown and release of cellular contents to the surrounding tissue, often triggering a dangerous inflammatory response [106].) Because cell death occurs before DNA repair is completed, this phenomenon could be seen as a way for multicellular organisms to quickly eliminate heavily damaged cells
rather than risk the error-prone and energy-intensive repair process [107]. PARP-mediated “cellular suicide” was eventually implicated in a range of pathologies, and the connection seemed to be supported by multiple studies identifying PARP inhibition as an effective therapeutic strategy in experimental models of diabetes [108, 109], inflammation [110], shock [111, 112], and neuronal death [113].

However, subsequent investigations have made it apparent that there is likely more to this story than simple NAD$^+$ depletion, and evidence is building that the suicide hypothesis is an inadequate explanation for PARP-1’s role in cell death. *In vitro* experiments have demonstrated that while PARP-1 inhibition is protective against cell death following DNA damage, this protection is not always accompanied by a reduction in NAD$^+$ consumption [83]. Furthermore, cells overexpressing PARP-1 have increased sensitivity to DNA damaging agents but exhibit neither NAD$^+$ depletion nor ATP starvation [114]. Ironically, it has even been found that ATP can be synthesized from PAR; thus, PAR produced in the aftermath of DNA damage may actually provide a local source of energy to fuel the necessary repairs [115]. *In vivo* work using animal models of various pathologies including cerebral ischemia, chronic colitis, and hemorrhagic shock has also raised concerns about the veracity of the suicide hypothesis; in each case, PARP inhibition clearly provides therapeutic benefits but these effects cannot be connected to the status of the cellular NAD$^+$ pool [104]. Furthermore, PARP-1 activation has been observed to occur during apoptosis [116–119], despite the fact that cell death as explained by the suicide hypothesis would be necrotic. Overall, the data suggest both that *in vitro* studies exaggerate the NAD$^+$ depletion resulting from PARP-1 activation, and that the beneficial effects of PARP-1 inhibitors may not be solely attributable to energy failure. It is possible that the suicide hypothesis applies only in situations of massive DNA disruption at levels not often seen *in vivo*. This hypothetical phenomenon has been referred to as “programmed necrosis [47].”

PARP-1’s emergence in the early 2000’s as a major transcriptional regulator brought to light other possible explanations for its role in cell death. As PARP-1 has been shown to both regulate chromatin accessibility and activate various transcription factors via either poly(ADP-
ribosylation or protein-protein interaction (which could be disrupted by excessive PAR), it is conceivable that DNA damage-mediated PARP-1 overactivation could perturb normal transcription in damaged cells and lead to necrosis and/or apoptosis [120]. There is also evidence that PARP-1 and/or PAR participate in specific inflammatory pathways; for example, inhibition of PARP-1 via either chemical inhibition or gene deletion reduces the activity of NF-κB, a transcription factor critical in immune and stress responses [111, 121, 122]. This in turn curbs the release of cytotoxic inflammatory cytokines and is protective against cell death in a number of disease models including stroke, septic shock, diabetes and Alzheimer's disease [123, 124]. The exact mechanism by which PARP-1 regulates NF-κB is not yet understood and there is some disagreement as to whether the process requires poly(ADP-ribosyl)ation activity [125, 126]; however, the two proteins have been shown to co-precipitate [127], indicating that protein-protein interactions may be involved.

Similarly, PARP-1 appears to play an important role in the modulation of p53, a transcription factor involved in cell cycle regulation and cell death [104]. p53 functions as a tumor suppressor by mediating G1 or G2 cell cycle arrest and allowing time for DNA repair prior to S phase, or if DNA damage is extensive enough, triggering apoptosis [128]. PARP-1 both poly(ADP-ribosyl)ates and participates in protein-protein interactions with p53 [129–134] and is in fact required for normal p53 activity [135, 136]. This effect may be explained in part by PARP-1-mediated stabilization of p53; cells from PARP-1 knockout animals have basal p53 levels up to twofold lower than normal [137]. But even in cells that are wild-type for PARP-1, chemical inhibition of the enzyme prevents p53 accumulation and activation of downstream targets in response to DNA damage, indicating that poly(ADP-ribosyl)ation is essential to p53’s role as an effector of cell cycle arrest and apoptosis [136]. It is interesting to note that p53 expression and activation are also dependent upon NF-κB [138–140]; thus, the interrelated pathways of PARP-1, NF-κB, and p53 comprise part of a finely-tuned system for the transcriptional regulation of cell death.

Finally, it has come to light that the PAR molecule itself is directly involved in triggering
cell death. When the Dawson group uncoupled PAR production from cellular NAD$^+$ and ATP levels via the direct introduction of in vitro synthesized PAR to living cells using a lipid-based delivery system, cell death occurred in a dose-dependent fashion, with long, complex polymers exhibiting the highest toxicity [141]. While this PAR-mediated cell death did not appear to demonstrate hallmarks of necrosis, it was also shown to be caspase-independent [142], unlike traditional apoptosis. This was a major indication that PARP-1 dependent cell death may comprise a novel signaling pathway. As apoptosis-inducing factor (AIF)-dependent cell death is also caspase-independent, the possibility of a potential connection between PARP-1 and AIF arose. AIF is a mitochondrial flavoprotein which normally plays a role in both oxidative phosphorylation and the maintenance of mitochondrial structure [143]. In response to death stimuli such as apoptosis-inducing drugs or transcription factors, however, AIF translocates to the nucleus due to permeabilization of the outer mitochondrial membrane. Once there, it induces chromatin condensation and DNA fragmentation, two morphological hallmarks of apoptotic cell death [144]. Multiple studies have demonstrated that the release of AIF from the mitochondria is PAR-dependent; chemical inhibition or genetic knockout of PARP-1 in cells treated with DNA-damaging agents prevents AIF translocation and rescues apoptosis [142, 145–147]. The exact mechanism by which PAR formation induces mitochondrial AIF release is not yet understood; however, it is known that PAR translocates from the nucleus to the cytoplasm during the process [147]. This type of cell death has been given the name “parthanatos” — from par for poly(ADP-ribose) plus thanatos, a Greek term for the philosophical notion of death — to distinguish it from both necrosis and caspase-dependent apoptosis [148]. The discovery of parthanatos has strengthened the notion that the “programmed necrosis” suggested by the suicide hypothesis may actually represent a disease state rather than a true pathway; it is likely that PARP-1 mediates cell death mainly through modulation of AIF and transcriptional control.

**Epigenetic control by PARP-1.** The term “epigenetics” refers to processes that modify the functional state of DNA without altering its sequence, including phenomena such as DNA
methylation and the posttranslational modification of histone tails [149]. Epigenetic changes generally affect gene expression by modulating the structure and accessibility of chromatin. It has been known since the early days of poly(ADP-ribosyl)ation research that PARP-1 activation is capable of effecting profound changes upon chromatin structure \textit{in vivo}; poly(ADP-ribosyl)ated nucleosomes have an open, relaxed configuration compared to their tightly-packed toroidal native state [73]. Furthermore, many of the proteins initially identified as targets of poly(ADP-ribosyl)ation, including histones and DNA ligases and polymerases [150], are involved in processes requiring the alteration of chromatin structure. These findings suggested a role for PARP-1 in epigenetic control. However, it was not until relatively recently that this idea was verified \textit{in vivo}. Work done in the model organism \textit{Drosophila melanogaster}, whose genome contains a single homolog to mammalian PARP-1, showed that PARP was responsible for the regulation of chromatin structure during development in oocytes and early embryos [151]. Specifically, PARP modulated both the expression and silencing of various euchromatic and heterochromatic regions throughout the developmental process, and PARP mutants displayed dramatically altered heterochromatin patterns as well as larval lethality. Subsequent studies involving fluorescence microscopy of the \textit{Drosophila} polytene chromosome gave a striking visualization of PARP-mediated chromatin remodeling at work [152]. Under normal circumstances PARP was found to be widely distributed along the chromosome, and largely inactive. After heat exposure, however, PARP accumulated rapidly at heat shock response loci and abundant PAR synthesis was detected. This PAR synthesis was associated with a visible loosening of chromatin in the affected regions, referred to as “puffs,” which were completely absent in the presence of a PARP inhibitor. In addition to confirming a role for PARP in epigenetic control, these data provided a convincing example of PARP activation by stimuli other than DNA damage.

The finding that PARP plays a role in gene silencing as well as activation was somewhat surprising considering its reputation as an effector of chromatin accessibility. Additional experiments by Tulin \textit{et al.} showed that an enzymatically inactive form of PARP could still modulate
heterochromatin formation during *Drosophila* oogenesis and larval development, raising the possibility that chromatin silencing by PARP, unlike chromatin activation, occurs via protein-protein or protein-DNA interactions independent of poly(ADP-ribosylation). This suggestion has been supported by recent studies involving atomic force microscopy of reconstituted chromatin, which demonstrated that a functional interaction between the DNA-binding and catalytic domains of PARP-1 induces nucleosome binding and leads to chromatin condensation and transcriptional repression, both of which are reversed upon PARP-1 automodification [153]. The dual effects of PARP-1 upon transcription have also been demonstrated in *PARP-1* knockout mouse fibroblasts; expression microarray analysis of these cells indicated reduced expression of a group genes involved in regulation of cell cycle progression, mitosis, DNA replication, and chromosome assembly, but elevated expression of a group of genes encoding extracellular matrix and cytoskeletal proteins that have been implicated in cancer and premature aging [135]. Thus, PARP-1 can modulate chromatin structure to be either more or less permissive of transcription depending upon the circumstances. It has been suggested that the local NAD$^+$ concentration is an important factor in determining whether PARP-1 behaves as a transcription activator or repressor [154], but it is not yet known how NAD$^+$ levels in particular regions of chromatin are regulated. Modulation of the enzymes involved in the NAD$^+$ synthesis pathway [155] and/or other chromatin-associated NAD$^+$-consuming enzymes such as the histone deacetylase SirT1 [156] are both interesting possibilities that require further investigation [154].

Lastly, there is now abundant evidence that PARP-1 is involved in the determination of DNA methylation patterns. DNA methylation, in which methyl groups are added to cytosines mainly cytosine-phosphate-guanine (CpG) dinucleotides by DNA methyltransferases (DNMTs), is the only known physiological postsynthetic modification of DNA that has epigenetic effects [106]. It is associated with inactive, condensed chromatin; only 1 to 2 percent of the genome is unmethylated at any given time, and these unmethylated CpGs are generally concentrated in the promoter regions of housekeeping genes [149]. Because the expression of many tumor suppressor genes is dependent upon the unmethylated state of CpG regions
(known as “CpG islands”) in their promoters, hypermethylation in these regions can cause genomic instability and cancer [149, 157]. On the other hand, hypomethylation of bulk genomic DNA can also have similar negative effects [158–160]. Maintenance of proper DNA methylation patterns is therefore of vital importance, and aberrant methylation is common in tumor cells. In the late 1990s, the Caiafa group began investigating a possible role for PARP-1 in this aspect of epigenetic control and discovered that chemical inhibition of PARP leads to hypermethylation of genomic DNA, including the CpG islands, in mouse fibroblasts [161, 162]. As DNMT mRNA and protein levels in cells treated with the PARP inhibitor 3-aminobenzamide are 2 to 4 times those of untreated cells, it was initially proposed that these effects result from increased DNA methyltransferase (DNMT) expression [163]. Subsequent work demonstrating that DNMT binds both PARP-1 and free PAR — and that these complexes are catalytically inefficient — suggests that modulation of DNMT’s enzymatic activity is also a factor [164]. In the current model, poly(ADP-ribosyl)ated PARP-1 competes with DNMT for DNA binding, reducing DNMT’s enzymatic activity and protecting CpG islands from methylation [165]. However, it is not yet understood how PARP-1 “knows” which DNA regions to mark for protection, and a concrete link between PARP-1 activity and hyper- or hypomethylation in human cancer is yet to be established.

**PARP-1 and Mammalian Cellular Aging: The Case for a Connection**

The correlation between DNA repair and mammalian longevity has long been recognized; not only do overall levels of DNA damage increase over a lifespan, but cells and organisms with greater DNA repair capacity tend to be longer-lived [166–169]. As DNA repair has been shown to become less efficient over time, the inability of the DNA repair machinery to maintain genome integrity in the face of ever-increasing amounts of DNA damage has been suggested as a major mechanism of the aging process [168]. This idea has been given credence by the discovery that
various human diseases of premature aging feature DNA repair deficiencies and progressive genome instability [170]. Considering that PARP-1 is an integral player in the DNA damage response, investigation of a possible link between PARP-1 and aging is a logical step. Along these lines, it has been reported that cellular poly(ADP-ribosyl)ation activity correlates with mammalian lifespan, and that lymphoblasts from human centenarians display higher PARP specific activity than those from control donors [171, 172]. However, this type of correlative evidence, while intriguing, is somewhat unsatisfying. There has been no experimental confirmation that PARP-1’s apparent relationship to aging and longevity is mediated by its involvement in DNA repair rather than some other pathway or cellular process. Furthermore, reasons for the observed variation in PARP specific activity amongst human individuals are not understood; the several known PARP-1 genetic polymorphisms have not been associated with longevity or poly(ADP-ribosyl)ation capacity [173], indicating that there must be other factors at play. We propose that PARP-1’s automodification status could be one of these factors, and therefore that characterization of the variation in PARP-1 automodification and enzymatic activity both in response to PARP-activating stimuli and during the course of cellular aging would provide valuable information about the regulation of the aging process.

We further hypothesize the direct participation of PARP-1 in mechanisms of cellular senescence. In light of the established correlation between PARP-1 and longevity, it is surprising that such a possibility has not yet been explored. Hayflick observed in 1965 that human diploid fibroblasts growing in culture undergo an initial period of rapid proliferation followed by a decrease in growth rate and an eventual cessation of replication now known as cellular senescence [174]. The induction of this non-proliferative state in cultured cells, whether through repeated passage [175] or addition of stress-inducing factors to the culture media, is widely used as a model for aging. While there has been some controversy regarding the direct applicability of in vitro senescence to organismal aging in vivo [176–178], the model clearly has value and has been indispensable in elucidating some of the cellular mechanisms underlying the aging process [179].
Correlative evidence indicates that a role for PARP-1 in cellular senescence is plausible. As discussed above, it is well-established that poly(ADP-ribosyl)ation can profoundly impact chromatin structure both \textit{in vitro} and \textit{in vivo}, contributing to either condensation and silencing [151] or relaxation and transcription [152] of chromatin depending upon the circumstances [59, 135]. Meanwhile, the importance of epigenetic control and chromatin remodeling in the establishment of senescence is quickly emerging. Narita \textit{et al.} have described the accumulation of distinct heterochromatin domains known as senescence-associated heterochromatin foci (SAHF) in senescent human fibroblasts. Formation of these foci depends upon the retinoblastoma (Rb) tumor suppressor, which is recruited along with various heterochromatin proteins to promoters regulated by the transcription factor E2F. The end result is stable silencing of E2F-responsive genes, many of which are required for cell cycle progression [180]. Formation of SAHF occurs in a stepwise manner dependent upon sequential post-translational modification and recruitment of various protein components [181–183]. Interestingly, SAHF contain enriched levels of the histone variant MacroH2A [181], which features an ADP-ribose-binding domain that forms strong associations with PAR \textit{in vitro} [34, 184]. Furthermore, another component of SAHF, the high-mobility group proteins [185], has been shown to be poly(ADP-ribosyl)ated [186, 187]. These findings suggest that PARP-1 could be involved in epigenetic regulation during senescence, and that variations in PARP-1 automodification status and/or enzymatic activity, as well as the extent and nature of cellular PAR, occur during cellular aging. The goal of this research is to identify some of these variations, paving the way for more detailed dissection of the enzyme’s role in this process.

**A True Understanding of Poly(ADP-ribosyl)ation Will Require Better Tools**

PARPs are commonly described as covalently modifying their targets [67]. Upward of 200 nuclear proteins have been identified as acceptors of poly(ADP-ribose), and covalent association
is usually assumed. However, careful interpretation of the literature actually reveals little evidence of covalent linkage [47]. Several ambiguities inherent in typical methods of poly(ADP-ribosyl)ation detection have contributed to this confusing state of affairs, including the fact that initial studies suggesting covalent modification were performed using relatively crude protein preparations [47]. For example, the assertion that PARPs covalently modify glutamate residues of their target proteins appears to originate with a pair of papers published in 1980 by Ogata et al. in which Glu-2 of histone H2B [9] as well as Glu-2, Glu-14, and the C-terminal lysine of histone H1 [10] were identified as ADP-ribose acceptor sites by proteolytic analysis of rat liver chromatin that had been incubated with radiolabeled NAD$^+$. Given the limitations of these studies it is surprising that their conclusions continue to be widely applied to PAR-accepting proteins almost 30 years later, especially when one considers the fact that experiments using highly purified PARP-1 rather than whole chromatin produced no proof of covalent histone modification [50, 54, 188].

As mass spectrometry has proven an invaluable tool for the determination of post-translational modification sites [189], it seems a natural choice for the confirmation of covalent protein modification by PARP-1. However, at the time this research was undertaken, analysis of the mono-ADP-ribosyltransferase ART2 [190] was the only convincing mass spectrometric identification of covalent poly(ADP-ribosyl)ation; such studies had not been successfully done with any PARP family member. This is due not to subpar effort on the part of PARP researchers, but rather to the fact that several biochemical properties of PAR — including its high mass, abundant negative charge, and variable structure — introduce major barriers to successful analysis. The dearth of qualitative evidence for PARP-mediated covalent poly(ADP-ribosyl)ation precludes more targeted studies such as site-directed mutagenesis, which could prove illuminative in the elucidation of PARP-1’s cellular functions. Accordingly, it has been said that the definitive demonstration of covalent poly(ADP-ribosyl)ation by PARPs would “undoubtedly change the field,” [47]. The further goal of this research is to develop methods for the unambiguous analysis of poly(ADP-ribosyl)ation both in vitro and in human fibroblasts during
oxidative stress and aging/senescence. These data would contribute toward the eventual elucidation of the putative “poly(ADP-ribose) code” [47] which may govern the cellular response to different types of PARP activity.

**Research Aims**

While there is much correlative evidence to suggest a connection between poly(ADP-ribose) (PAR) and mammalian longevity, no studies have been done to explore a possible role for PARP-1 — the enzyme responsible for synthesis of 90% of cellular PAR — in mechanisms of senescence. Furthermore, many techniques currently being used for analysis of protein poly(ADP-ribosyl)ation are fraught with imprecision. We therefore sought to address these issues by refining methods for the quantitative analysis of poly(ADP-ribosyl)ation both *in vitro* and in cultured human cells, and using those methods to evaluate our hypothesis that PARP-1 is a player in mechanisms of cellular senescence. A graduate fellowship was awarded by the National Institute on Aging to support these efforts. Aims of the research project were as follows:

1. **Use an *in vitro* system to develop biochemical and mass spectrometric methods for the unambiguous analysis of poly(ADP-ribosyl)ated proteins.** Due to the challenges introduced by PAR's biochemical characteristics, successful mass spectrometric analysis of poly(ADP-ribosylation) will require the use of techniques to reduce the mass, charge, and heterogeneity of the polymer. To this end, the effectiveness of partial degradation of PAR with snake venom phosphodiesterase (SV-PDE) as well as manipulation of poly(ADP-ribosyl)ation reaction conditions to favor the formation of short rather than long polymer chains was evaluated. The use of ammonium sulfate fractionation, boronate affinity chromatography, and IMAC to enrich samples for poly(ADP-ribosyl)ated protein was also explored. These techniques were applied *in vitro* to both trans-(ADP-ribosyl)ated histone and auto(ADP-ribosyl)ated PARP-1, and materials were analyzed as both whole proteins and tryptic digests using MALDI and Q-TRAP mass spectrometry. Enzymatically active recombinant human PARP-1 was expressed and purified for
2. Test the hypothesis that PARP-1 post-translational modifications, protein levels, and/or enzymatic activity are involved in processes of cellular aging and senescence. Past work in our laboratory has demonstrated that nicotinamide (NAM), a component of vitamin B₃, significantly extends the replicative lifespan of human fibroblasts [191]. The perturbation of senescence processes by NAM treatment could be a useful tool for the elucidation of PARP-1’s connection to cellular aging; while NAM is a weak PARP-1 inhibitor [192], there are many other means by which PARP-1 could be involved in NAM-mediated lifespan extension. NAM has been shown to regulate a wide range of cellular processes, including metabolism and inflammation, by mechanisms that are not well understood [193]. It is therefore likely that NAM treatment during the aging of human fibroblasts would alter PARP-1 levels and/or enzymatic activity as compared to untreated cells. We have characterized these differences in proliferating and senescent human fibroblasts in the presence and absence of NAM. Senescence markers including reduced cellular proliferation, senescence-associated heterochromatin foci (SAHF), and senescence-associated β-galactosidase activity (SA-β-gal) were also monitored to give a more complete picture of cellular “age.”
Methods Development for Mass Spectrometric Analysis of Poly(ADP-ribosyl)ation

Introduction

What Do We Really Know About Protein Poly(ADP-ribosyl)ation?

While PARP’s role in important cellular processes such as DNA repair, mediation of cell death, and epigenetic control has been the focus of intense research for over 40 years, one of the enzyme’s most basic defining characteristics — that it modifies acceptor proteins, including itself, on glutamate residues — is widely accepted today largely upon the basis of two small sets of experiments done in the early 1980s. Using radiolabeled NAD and a proteolytic digestion approach, Ogata et al. identified Glu-2 of histone H2B and Glu-2, Glu-14 and the C-terminal lysine of histone H1 as the ADP-ribose-accepting residues in chromatin isolated from rat liver [9, 10]. These experiments were performed using a crude chromatin preparation rather than pure proteins, and the modifications analyzed were almost certainly mono- rather than poly(ADP-ribosyl)ation, as studies done with highly purified PARP-1 have consistently failed to demonstrate covalent histone PARylation [50, 54, 188]. Nevertheless, the subsequent discovery that the PAR-PARP linkage is chemically similar to the glutamate carboxyl esters found in mono-ADP-ribosylated histone [61], in conjunction with reports suggesting that PARP-1 is au-
tomodified on 4 to 28 acceptor sites in its glutamate-rich automodification domain [61, 62, 194], led to speculation that glutamate is the protein residue targeted by PARP-1 [195]. This hypothesis came to be treated as a settled matter despite the fact that not one of these purported PAR-accepting glutamate residues could be definitively identified, on PARP-1 or any other protein, over decades of research. The oft-repeated claim that PARP-1 modifies glutamate residues therefore does not appear to be based upon scientific evidence.

The assumed covalent nature of protein poly(ADP-ribosyl)ation must similarly be called into question. More than 200 nuclear proteins have been suggested as targets of PARP-1 (Table 1); however, studies claiming to demonstrate covalent linkage have relied on such techniques as co-immunoprecipitation or the assembly of in vitro poly(ADP-ribosyl)ation reaction mixtures containing PARP plus the protein of interest, followed by Western blotting or autoradiography. This is problematic because PAR has been found to form strong, stable noncovalent interactions with various proteins, including many of its purported targets such as histones [196, 197], p53 [198], and other nuclear proteins [32, 198]. These interactions are highly specific and resistant to salt, acid, detergent [47], and even electrophoresis [33]. One can thus not prove covalent protein-PAR interaction in this fashion.

In sum, we are left with a situation in which complex studies of PARP-1’s cellular functions are being carried out while some of the most very basic questions about the enzyme remain unanswered. The current inability to definitively identify sites of covalent poly(ADP-ribosyl)ation is frustrating not only for this reason, but because it prevents the execution of studies such as site-directed mutagenesis which would likely prove helpful in further elucidating the exact mechanisms behind PARP’s many cellular roles. According to leaders in the field, this situation “has to be urgently addressed” [47].
<table>
<thead>
<tr>
<th>Substrate(s)</th>
<th>Proposed functional relevance</th>
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<tbody>
<tr>
<td>Histones</td>
<td>Inhibition of DNA binding activity</td>
</tr>
<tr>
<td>High-mobility-group proteins</td>
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<tr>
<td>Low-mobility-group protein</td>
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<tr>
<td>Poly(ADP-ribose) polymerases</td>
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</tr>
<tr>
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<tr>
<td>Topoisomerase I and II</td>
<td>Inhibition of catalytic activity</td>
</tr>
<tr>
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<tr>
<td>CENPA, CENPB, Bub3</td>
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<tr>
<td>p53</td>
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<tr>
<td>PCNA</td>
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<tr>
<td>Telomeric repeat binding factor-1</td>
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<tr>
<td>CTCF</td>
<td>Enhancement of DNA binding</td>
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<tr>
<td>NuMa</td>
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</table>

* NA, not analyzed.
Mass spectrometric analysis of poly(ADP-ribosylation): complications and possibilities.

Because post-translational modifications (PTMs) cause an increase in mass compared to the expected molecular weight of a protein based on its sequence, mass spectrometry is generally considered an excellent tool for PTM analysis. Accordingly, the aim of this work is to develop methods for the mass spectrometric analysis of poly(ADP-ribosylation). It is clear, however, that this will be no easy task; thus far there has never been an unambiguous identification of covalent poly(ADP-ribosyl)ation by PARP-1 despite years of effort by various researchers in the field (reviewed in [47]). These difficulties can be attributed to several complicating biochemical properties of the PAR polymer. Firstly, PAR's multiple phosphate groups give it abundant negative charge. Poly(ADP-ribosylated) peptides are thus less likely to be detected by a mass spectrometer running in positive mode, which is the standard ionization mode for peptide analyses. (Negative mode ion detection is possible, but interpretation of the resulting spectra is difficult and has not been extensively investigated [199].) The polymer's substantial mass also presents difficulties; attachment of one ADP-ribose unit causes a mass increase of 541, and polymer chains upward of 200 units in length have been described. The addition of such a large modification to a protein or peptide would likely interfere with ionization processes, again reducing the chance of detection during mass spectral analysis.

Other challenges in the characterization of poly(ADP-ribosylation) by mass spectrometry lie in PAR's structural heterogeneity. Poly(ADP-ribosylation) produces a series of molecules that are derived from the same peptide but differ greatly in both chain length and branching, resulting in a heterogeneous distribution of peptide isoforms. To make matters worse, the polymer may fragment at labile bonds during ionization (in-source decay), further increasing sample complexity (Figure 4). Thus, even if poly(ADP-ribosyl)ated peptides are present in a mixture and successfully ionized, the signal intensity of any particular modified species may not reach detectable levels. Finally, the commonly used algorithms for peptide identification are not very effective when modified peptides of unknown structure or site of attachment are present.
Combined, these issues have proven sufficient to prevent qualitative mass spectrometric analysis of poly(ADP-ribosyl)ation. Tackling of this problem will therefore require the application of novel combinations of biochemical and mass spectrometric techniques to reduce the mass, charge, and heterogeneity of the polymer, as well as methods to enrich methods for poly(ADP-ribosyl)ated protein. Several approaches that will be explored in this work (Table 2) are discussed below; we will evaluate the effectiveness of these techniques for sample preparation of histones and PARP-1 poly(ADP-ribose)lated in vitro prior to MS analysis.

**Ammonium sulfate fractionation.** Ammonium sulfate fractionation is a method for the purification of proteins based on relative solubility. When a native protein is in solution, its hydrophobic side chains are generally sequestered in the interior of the structure with the charged and hydrophilic side chains on the structure's exterior. These side chains can participate in salt bridges with nearby residues as well as hydrogen bonds with water. Increasing the ionic strength of the solution reduces the availability of these water molecules for hydrogen bonding; the protein's charged side chains are then increasingly forced to participate in interactions with other amino acid residues instead, resulting in protein precipitation. While this technique (also known as “salting out”) may be performed with a variety of salts, ammonium sulfate is a
good choice due to its high solubility in water [200]. We propose a new utility for ammonium sulfate precipitation in the enrichment of samples for poly(ADP-ribosyl)ated protein. Post-translational modification status is known to alter protein solubility; it therefore follows that the addition of PAR chains could shift the ammonium sulfate fraction at which a protein precipitates during salting out. We will explore the utility of ammonium sulfate precipitation in separating poly(ADP-ribosyl)ated from unmodified protein.

**Boronate affinity chromatography.** Immobilized boronates were first used for the separation of compounds containing 1,2 cis-diol groups by Weith *et al.* in 1970 [201]. Since then, boronate affinity resins have been developed for the purification of nucleic acids, sugars, and glycoproteins [202]. The key interaction in this type of chromatography is the formation of an ester bond between the boronate ligand and the cis-diol; although the bonds formed are covalent, they are readily hydrolyzed under acidic conditions in aqueous solution, allowing release and collection of the purified material with a simple acidic wash [203]. Poly(ADP-ribosyl)ated proteins should form strong interactions with boronates due to the 1,2 cis-diol found in the ribose moiety of PAR. We will therefore evaluate the effectiveness of boronate affinity chromatography in reducing sample complexity by enriching samples for poly(ADP-ribosyl)ated protein.

"Solid-state" poly(ADP-ribosyl)ation of immobilized proteins. Because the primary acceptor of poly(ADP-ribose) is PARP-1 itself, the experimental use of traditional soluble *in vitro* poly(ADP-ribosyl)ation reactions requires the analysis of a complicated mixture containing the modified protein of interest (trans-ADP-ribosylation), modified PARP-1 (auto-ADP-ribosylation), and most likely, free PAR. Kirsten *et al.* [204] have suggested that the use of a “solid state” system in which the protein of interest is immobilized to 96-well plates would easily allow the physical separation of trans- and automodified protein; supernatants containing free PAR and automodified PARP-1 can be removed, while the well-bound material containing transmodified protein can be analyzed separately. We will use this approach to study poly(ADP-ribosyl)ation of immobilized histone H1.
Digestion of poly(ADP-ribose) with snake venom phosphodiesterase. Snake venom phosphodiesterase (SVPDE) was an important tool in the early days of PARP research; as SVPDE treatment readily cleaves the pyrophosphate linkages of PAR, techniques involving digestion of poly(ADP-ribosyl)ated material followed by chromatographic and/or electrophoretic analysis of the reaction products were instrumental in determining the polymer's structure [2, 5]. While PAR structure is a now a settled matter, we envision an additional use for SVPDE in the analysis of poly(ADP-ribosyl)ation using mass spectrometry. Partial digestion of protein-associated PAR chains would reduce both mass and charge, increasing the chances that a poly(ADP-ribosyl)ated peptide would be successfully ionized and detected during mass spectrometry. But moreover, complete hydrolysis of the polymer — which can be accomplished with two hours of SVPDE treatment at 37 °C [39] — would theoretically leave the final ribose-5-phosphate unit attached to the protein as a natural tag. The addition of a 5-phosphoribosyl group to the carboxylic acid side chain of glutamate would increase the mass of the residue by 212 amu, a mass increase that proteomics search algorithms are capable of employing for peptide identifications. We will test the effectiveness of SVPDE treatment in sample preparation of poly(ADP-ribosyl)ated histone H1 for MS analysis.

Manipulation of PARP-1 reaction conditions to favor the generation of short poly(ADP-ribose) chains. Though SVPDE treatment is a time-tested method for the degradation of PAR, it could complicate analysis by introducing another protein to the mix. We will therefore explore other means of reducing polymer mass/charge and sample complexity without the use of additional enzymes. Several studies have demonstrated that the length of PAR chains synthesized during in vitro PARP-1 reactions is highly dependent upon the substrate concentration; the use of nanomolar rather than micromolar NAD concentrations causes a dramatic shift in the overall character of the resulting polymers, producing short oligomers of 1, 2 or 3 units [205, 206]. This would substantially reduce both polymer mass and sample complexity by eliminating the presence of long and/or branched PAR chains. Using this approach, we will prepare samples
of short-chain automodified PARP-1 to determine whether MS analysis of this material is more illuminative than that of typical long-chain poly(ADP-ribosyl)ated protein.

**Immobilized Metal Ion Affinity Chromatography.** Immobilized metal affinity chromatography (IMAC), also known as metal chelate chromatography, is the separation of proteins on the basis of differential interaction with chelated metals [207]. Amino acid composition is a major determinant of a particular protein’s affinity for the resin; side chains such as histidine, tryptophan, and cysteine interact with transition metal ions bound to the column and contribute to increased protein retention, after which the purified material may be eluted by lowering the pH. Post-translational modification status can also have a dramatic impact on protein binding. Consequently, IMAC is commonly used today for the purification of phosphopeptides [208]. As PAR contains multiple phosphate groups and is highly negatively charged, we speculate that IMAC affinity resins designed for phosphopeptide separation may also retain poly(ADP-ribosyl)ated protein. To test this hypothesis, we will employ IMAC to separate a tryptic digest of automodified PARP prior to mass spectrometric analysis.

**Table 2.** Summary of sample preparation approaches that will be evaluated for mass spectrometric analysis of poly(ADP-ribosyl)ation. The particular difficulties that each method is intended to address are indicated with checkmarks.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Reduction of sample complexity</th>
<th>Reduction of PAR chain length</th>
<th>Enrichment for PARylated proteins</th>
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<td>Ammonium sulfate fractionation</td>
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<tr>
<td>Boronate affinity chromatography</td>
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<tr>
<td>SVPDE digestion</td>
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<tr>
<td>Poly(ADP-ribosylation) with nM NAD</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>IMAC</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>
Experimental Procedures

Materials and reagents. Unless stated otherwise, reagents were purchased from either Sigma-Aldrich or Fisher Scientific. Cell culture reagents were purchased from Invitrogen, ATCC, or Atlanta Biologicals. Flasks and plates for cell culture were supplied by Corning. All cell lines were obtained from ATCC. End-protected DNA 23-mers (fluorescein-5’-CAAGTGTTCATTCTCTCCTGGG-3’-biotin and its reverse complement) for in vitro poly(ADP-ribosyl)ation reactions [56] were synthesized by Midland Certified Reagent Company. Restriction enzymes were supplied by New England Biosystems. Gateway-compatible PCR primers were designed according to Invitrogen’s guidelines using the Primer3Plus web interface (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by Midland Certified Reagent Company. All other Gateway cloning materials were purchased from Invitrogen.

Mammalian cell culture, PARP-1 stimulation, and cell harvesting. All cell manipulations were performed in a laminar flow hood under aseptic conditions. U2-OS human osteosarcoma cells were grown in 150 cm² tissue culture flasks and maintained in a 37° C incubator at 5% (v/v) CO₂ in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Medium was replaced every 3-4 days. Cells were subcultured at 70-85% confluency by decanting the medium, rinsing the flask with PBS, and adding 0.25% (w/v) trypsin-EDTA (2-3 mL, prewarmed to 37° C). The cells were incubated at 37° C for 5-10 minutes until cell detachment occurred. Trypsin was neutralized by addition of supplemented DMEM to a final volume of 10 mL and cells were seeded in fresh flasks at a 1:3 to 1:8 ratio.

To stimulate PARP-1 activity, growth medium was removed and replaced with serum-free DMEM containing 0.5 mM H₂O₂ for 20 min. Medium was then discarded and cells were harvested by scraping into ice-cold PBS, transferred to a 15 mL centrifuge tube, and centrifuged at 300 x g for 5-10 min in order to pellet the cells and decant the PBS. The cell pellet was subsequently washed with an additional 10 mL of PBS, centrifuged, and decanted as described above.
before storing the tube on its side at -80°C until further analysis.

**Cell extract preparation.** Whole cell lysates were prepared by resuspending U2-OS cell pellets in 1 mL of either NET-N buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP-40) or EBC buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP-40) and incubating on ice with occasional agitation for 30 min. Proteases were inhibited during lysis using Roche’s Complete Mini Protease Inhibitor Cocktail Tablets according to manufacturer’s instructions. Protein concentrations were determined by the Bradford method [209] with a BSA standard using Pierce’s Coomassie Plus reagent kit.

**Ammonium sulfate fractionation.** U2-OS whole cell lysates (1 mg) were brought to a volume of 1 mL with PBS and placed in an Eppendorf tube on ice. For each precipitation step, solid (NH$_4$)$_2$SO$_4$ was slowly added with constant stirring. Stirring was then continued for an additional 30 min after which the precipitate was pelleted by centrifugation at 4 °C for 20 min at 20,000 x g. The supernatant was returned to the tube and the steps were repeated until an (NH$_4$)$_2$SO$_4$ concentration of 80% (w/v) had been reached. Grams per liter of (NH$_4$)$_2$SO$_4$ to add for each precipitation step [200] were calculated using the formula $g/L = \frac{533(S_2-S_1)}{100-0.3(S_2)}$ where $S_1$ = the original salt concentration and $S_2$ = the final salt concentration. Actual amounts are shown in Table 3. Pellets were resuspended in 100 μL PBS. Residual (NH$_4$)$_2$SO$_4$ was removed from the resolubilized pellets and supernatant by dialysis into 5 L of PBS using 5k MWCO Slide-A-Lyzer Mini units (Pierce). Dialysis was performed at 4°C for 1.5 hours followed by an additional 1.5 hours and then overnight; buffer was changed after each step. Protein concentrations were determined by the Bradford method [209] with a BSA standard using Pierce’s Coomassie Plus reagent kit, and samples were stored at -20 °C for later analysis by Western blot.

**Protein electrophoresis, Coomassie staining and Western blotting.** Cell lysates containing equal amounts of protein (15-50 μg) in Laemmli SDS-PAGE sample buffer (with 2.5% (v/v) β-mercaptoethanol) were heated for 5 min at 95 °C and loaded on a 4-15% Tris-HCL gel (Bio-
Table 3. Ammonium sulfate precipitation scheme.

<table>
<thead>
<tr>
<th>% Salt (w/v)</th>
<th>(NH₄)₂SO₄ (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.121</td>
</tr>
<tr>
<td>60</td>
<td>0.130</td>
</tr>
<tr>
<td>80</td>
<td>0.140</td>
</tr>
</tbody>
</table>

Rad). Electrophoresis was performed for 45-60 minutes at constant voltage (200 V) using a Mini-PROTEAN cell and PowerPac power supply (Bio-Rad).

For Coomassie staining, the gel was immersed in stain solution (0.025% (w/v) Coomassie Brilliant Blue, 40% (v/v) methanol, 7% (v/v) acetic acid) with gentle agitation for 1-4 hours, then in destain solution (40% (v/v) methanol, 7% (v/v) acetic acid) until background was reduced and protein bands were clearly visible. The gel image was then captured on a Molecular Imager GS-800 densitometer (Bio-Rad). Residual background staining was removed from the image using Quantity One software (Bio-Rad).

For Western blotting, proteins were transferred to a Hybond-P PVDF membrane (GE Healthcare) using a Mini-Trans-Blot cell and PowerPac power supply (Bio-Rad) for 2 hours at constant current (400 mA). The membrane was blocked in 5% (w/v) nonfat dry milk/TBS-T at room temperature for one hour. Primary antibody incubation (1:1000 dilution in 5% (w/v) milk/TBS-T) was performed for 3 hours at room temperature or overnight at 4º C using anti-PARP-1 C-2-10 mAb or anti-PAR mAb (BioMol). The membrane was washed in TBS-T (3 x 5 min) and then incubated with a goat anti-mouse HRP conjugated secondary antibody (Pierce, 1:5000 dilution in 5% (w/v) milk/TBS-T) for 1 hour at room temperature. Following three 5-minute washes in TBS-T, signal was developed using ECL-Plus (GE Healthcare) according to the manufacturer’s instructions and detected using X-ray film (Kodak) or a Gel-Doc XR+ imager (Bio-Rad).

Boronate affinity chromatography. To create boronate affinity spin columns, a slurry was first prepared by suspending 0.08 g of Affi-Gel Boronate Affinity Gel (Bio-Rad) in 800 µL of a 50:50 (v/v) mix of 100% methanol and boronate binding buffer (50 mM ammonium bicarbonate
pH 8.0, 6 M guanidine-HCl). Sixty µL of this slurry was added to mini spin columns (Pierce). Columns were centrifuged at 1000 x g for 1 minute, flow-through was discarded, and resin was washed with boronate binding buffer (4 x 100 µL).

Purified recombinant PARP-1 was buffer-exchanged into 50 mM ammonium bicarbonate (pH 8.0) using a centrifugal concentrator (10 kDa MWCO; Microcon, Millipore) according to the manufacturer’s instructions. Reaction mixtures containing 10 µg PARP-1 were assembled in 90 µL of PARP reaction buffer (50 mM ammonium bicarbonate pH 8.0, 25 mM MgCl₂, 10 nM dsDNA). To begin reactions, 10 µL of 1 mM NAD was added for a final concentration of 100 nM. Samples were incubated at room temperature for 10 minutes after which reactions were stopped by buffer exchange into 100 µL of fresh 50 mM ammonium bicarbonate (pH 8.0) using centrifugal concentration (10 kDa MWCO; Microcon, Millipore) and applied to the previously prepared boronate affinity spin columns. Columns were incubated at room temperature for 10 min with occasional gentle agitation after which flow-throughs were collected by centrifugation (1000 x g for 1 min) and reapplied to the columns. After an additional 10 min incubation, flow-throughs were collected by centrifugation and placed on ice. Columns were washed with boronate binding buffer (4 x 100 µL), bound material was eluted twice with 50 µL of boronate elution buffer (50 mM ammonium acetate pH 6.0, 6 M guanidine-HCl), and eluates were collected by centrifugation. To remove residual guanidine-HCl, flow-throughs and eluates were then dialyzed into 5 L of 6 M urea for 1 hour at room temperature using 10k MWCO Slide-A-Lyzer Mini dialysis units (Pierce). Samples were mixed with an equal volume of Laemmli SDS-PAGE sample buffer (Pierce) containing 5% (v/v) β-mercaptoethanol, heated at 95 ºC for 5 min, and stored at -20 ºC for later analysis by Western blot.

**Solid-state poly(ADP-ribosyl)ation and analysis of immobilized histone H1.** Lyophilized histone H1 from calf thymus (Sigma) was reconstituted to 100 µg/mL in PBS and added to a 96-well plate (50 µL/well). The plate was incubated overnight at 4 ºC, after which the histone solution was aspirated and the plate was washed thoroughly with PBS (5 x 100 µL/well, tapping plate
on a paper towel to virtual dryness between washes). PARP-1 reaction buffer (50 mM Tris-HCL pH 8.0, 400 μM NAD, 25 μM bio-NAD, 10 nM end-protected dsDNA) was added (45 μL/well) followed by PARP-1 (Trevigen, 5 μL/well of a 10 ng/μL solution for a final concentration of 8 nM) to begin the reactions. The plate was incubated for 10 min at room temperature, after which the supernatants were removed, snap-frozen in liquid nitrogen, and stored at -20 ºC for later analysis by Western blot. The plate was then washed with PBS (4 x 100 μL).

For LC-MS/MS, well-bound material was digested overnight in 100 μL of 25 mM ammonium bicarbonate pH 8 with trypsin added at a 1:50 (w/w) ratio. Samples were then analyzed using an UltiMate capillary HPLC system (LC Packings) interfaced with an LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan) in NSI mode. A peak list generated using the machine’s Xcalibur software was compared to a mass table of expected fragments from the N-terminal acetyl peptide of histone H1 generated with Mascot (www.matrixscience.com).

For colorimetric detection of PAR, the plate was blocked with 300 μL/well Starting Block (Pierce). A 1:500 (v/v) dilution of HRP-streptavidin (Pierce) in Starting Block was then added (50 μL/well) and the plate was incubated for 30 min at 37° C. The HRP-streptavidin solution was aspirated and the plate was washed extensively with PBS (5 x 100 μL/well). TACS-Sapphire peroxidase reagent (Trevigen) was added (50 μL/well) after which the plate was incubated in the dark for 10 min at room temperature. To stop the peroxidase reaction, samples were acidified with 0.2 N HCl (50 μL/well). Absorbance at 460 nm was read on a Tecan microplate reader.

**Preparation of poly(ADP-ribosyl)ated histone H1.** Reaction mixtures containing 50 μg histone H1 (Calbiochem) were assembled in 48 μL of PARP reaction buffer (50 mM Tris-HCL pH 8.0, 25 mM MgCl2, 100 μM NAD, 10 nM end-protected dsDNA). To begin reactions, 2 μL of 190.9 nM PARP-1 (Trevigen) was added for a final concentration of 8 nM. Samples were incubated at room temperature for 10 min after which reactions were stopped by addition of ice-cold TCA to a final concentration of 10% (v/v). Samples were vortexed and left at 4 ºC overnight, then centrifuged at 4 ºC for 15 min at 15,000 x g to pellet the precipitates. Supernatants were discarded
and pellets were washed twice in ice-cold 100% acetone, then air-dried. Pellets were then re-suspended in 48 µL of SVPDE reaction buffer (110 mM Tris-HCl pH 8.0, 110 mM NaCl, 15 mM MgCl₂). To begin reactions, 2 µL of SVPDE (0.0142 U/µL, Sigma) was added and samples were incubated at 37 ºC for 2 hours. Reactions were stopped by TCA precipitation, pelleted, washed, and dried as described above, after which the pellets were stored at -20 ºC until MS analysis.

Preparation of automodified PARP-1. Reaction mixtures containing 10 µg purified recombinant PARP-1 were assembled in 99 µL of PARP reaction buffer (50 mM ammonium bicarbonate pH 8.0, 25 mM MgCl₂, 10 nM dsDNA). To begin reactions, 1 µL of NAD (0.1 µM or 100 µM) was added for a final concentration of 100 nM (for short-chain PAR formation) or 100 µM (for long-chain PAR formation). Samples were incubated at room temperature for 10 min after which reactions were stopped by removal of NAD via buffer exchange into fresh ammonium bicarbonate using 10k MWCO Centricon centrifugal filter devices (Millipore) according to the manufacturer’s instructions. Total reaction time (room temperature incubation time plus time elapsed during buffer exchange) was approximately 15 min. Samples were snap-frozen in liquid nitrogen and stored at -20 ºC until MS analysis.

LC-MALDI and MALDI-TOF mass spectrometry. For LC-MALDI, protein samples were digested overnight in 100 µL of ammonium bicarbonate pH 8 (25 mM) with trypsin added at a 1:50 (w/w) ratio. Digests were brought to 0.2% (v/v) trifluoroacetic acid (TFA) using a 5% (v/v) stock solution and the pH was adjusted to below 3 using 98% (v/v) formic acid if necessary. Samples (10 µL) were loaded onto a 0.3 mm ID C18PM (LC Packings) precolumn using a TEMPO autosampler and nano MDLC unit (AB Sciex). The precolumn was washed for 15 min at 15 µL/min with water:acetonitrile (98:2 v/v) supplemented with TFA (0.1% v/v). The precolumn was then switched in-line with a 100 mm x 0.1 mm column containing Synergi 4µ Hydro RP 80Å bulk packing material (Phenomenex) packed in-house using a self-pack IntegraFrit capillary column (New Objective). Peptides were eluted at 700 nl/min using a 105 minute gradient from 100% solvent A (98:2 v/v water:acetonitrile supplemented with 0.1% v/v TFA) to 90% solvent B
(98:2 v/v acetonitrile:water supplemented with 0.1% v/v trifluoroacetic acid) as follows:

1. Linear gradient to 15% solvent B over 5 min;
2. Linear gradient to 45% solvent B over 40 min;
3. Linear gradient to 90% solvent B over 10 min;
4. Hold at 90% solvent B for 10 min;
5. Linear gradient to 0% solvent B over 5 min; and
6. Re-equilibration at 0% solvent B for 35 minutes.

Column effluent was mixed with matrix delivered via a syringe pump attached to an Ekspot LC-MALDI spotting robot (Eksigent) at a flow rate of 2 µL/min. Alpha-cyano-4-hydroxy-cinnamic acid (4 mg/mL) in 1:1 (v/v) water:acetonitrile supplemented with 0.2% (v/v) TFA and ammonium citrate dibasic (20 mM) was used as the matrix. The effluent/matrix mixture was then spotted at 30 seconds per spot onto an Opti-TOF 384 well MALDI insert (Applied Biosystems).

For MALDI-TOF/TOF peptide or protein identification, material spotted onto each spot of a MALDI insert whether previously separated via LC-MALDI (multiple spots per sample) or not (single spot per sample) was analyzed first in reflector positive ion mode using a 4800 MALDI TOF/TOF instrument (Applied Biosystems) for the m/z range from 800 to 4000 for tryptic digest (or 5000 to 35,000 for whole protein), typically averaging spectra obtained from 750 to 1000 laser shots. The top 7 to 10 peaks above a minimum signal to noise ratio (typically 25 to 35) were then submitted automatically via the mass spectrometer's 4000 Series Explorer software (v. 3.5.28193, build 1011) for MS/MS analysis in the 1kV positive ion mode. The 4000 Series Explorer software was then used to generate a peak list containing significant peaks from both MS and MS/MS.

For analysis of free PAR polymer, a commercial preparation of PAR (BioMol) containing polymers ranging from 2-300 units in length with an average length of 20 units was mixed 1:1 with a trihydroxyacetophenone matrix and spotted directly onto the MALDI plate. MALDI-TOF
analysis was then performed in negative reflectron mode and data was collected over the mass range of 200 to 8000.

**High-power MALDI MS analyses.** Protein samples were digested overnight in 100 µL of ammonium bicarbonate (25 mM, pH 8) with trypsin added at a 1:50 (w/w) ratio. Digests were brought to 0.2% (v/v) TFA using a 5% (v/v) stock solution and the pH was adjusted to below 3 using 98% (v/v) formic acid if necessary. A portion of the acidified digest was then manually loaded onto a C18 reversed phase trap cartridge using a syringe pump. The cartridge was washed with solvent A (2% v/v acetonitrile in water supplemented with 0.2% w/v TFA) to remove residual salts, then attached to an Eksigent nanoLC pump and placed in-line with a 10 cm x 100 µm C18 reversed phase nanocolumn. Peptides were eluted using a gradient from 100% solvent A to 100% solvent B (2% v/v water in acetonitrile supplemented with 0.2% w/v TFA) at a flow rate of 500 nL/min. The column eluate was mixed with a matrix solution delivered at 1 µL/min and drops of the mixture were spotted sequentially onto a MALDI target plate every 30 seconds. The matrix solution used was 5 mg/mL α-cyano-4-hydroxy-cinnamic acid (αCHCA) in 50:50 (v/v) water:acetonitrile supplemented with 0.2% (w/v) TFA and 20 mM ammonium citrate dibasic. Spots on the MALDI target plate were allowed to air dry, and data was collected in negative reflectron mode using an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer. Data was collected for 200 laser shots over the mass range of 50 to 1800 with a laser setting of 7200. The machine's 4000 Series Explorer software was then used to generate a chromatogram of the phosphate-, AMP-, and ADP-containing species.

**Q-TRAP LC-MS/MS.** Acidified tryptic digests (10 µl) were loaded onto a 0.3 mm ID C18PM (LC Packings) precolumn using a TEMPO autosampler and nano MDLC unit (AB Sciex). The precolumn was washed for 10 minutes at 10 µl/min with 98:2 (v/v) water:acetonitrile supplemented with 0.1% (v/v) formic acid. The precolumn was then switched in-line with a 100 mm x 0.1 mm column containing Synergi 4µ Hydro RP 80Å bulk packing material (Phenomenex) packed in-house using a self-pack IntegraFrit capillary column (New Objective). Peptides were eluted at
500 nl/min using a 60 minute gradient from 100% solvent A (98:2 v/v water:acetonitrile supplemented with 0.1% v/v formic acid) to 100% solvent B (98:2 v/v acetonitrile:water supplemented with 0.1% v/v formic acid) as follows:

1. Linear gradient to 20% B over 5 minutes;
2. Linear gradient to 65% B over 20 minutes;
3. Linear gradient to 100% B over 5 minutes;
4. Hold at 100% B for 15 minutes;
5. Linear gradient to 0% B over 5 minutes; and
6. Re-equilibration at 0% B for 10 minutes.

Column effluent was introduced into a 4000 Q-TRAP linear ion trap mass spectrometer (AB Sciex) via a MicroIon II nanospray source (AB Sciex). A linear ion trap enhanced mass spectrum (EMS) scan in positive ion mode was collected for m/z from 400-2400. Ions exhibiting an intensity greater than 360000 cps and bearing a charge state of either +2, +3, +4 or indeterminate as determined by an enhanced resolution (ER) scan were then analysed by an enhanced product ion (EPI) scan for m/z from 225-1515 using a variable collision energy dependent upon charge state and m/z value as shown in Table 4. The EMS scan speed was 4000 amu/sec, the ER scan speed was 250 amu/sec and the EPI scan speed was 1000 amu/sec with a fixed fill time of 25 ms. The EMS and ER scans utilized the linear ion trap’s dynamic fill time capability wherein a quick prescan determines the flux of ions to the trap prior to the scan. The target number of ions was $1.0 \times 10^7$ for the EMS scan and $0.5 \times 10^7$ for the ER scan. The minimum allowable fill time was 2 ms for both scans and the maximum allowable fill times for the EMS and ER scans were 150 and 250 ms, respectively. Other mass spectrometer parameters used were: curtain (CUR) and sheath (GS1) gases at 10 (arbitrary units), CAD gas at high, ion spray voltage at 2500V, declustering potential at 65 V, and interface heater temperature set at 160°C. Dynamic exclusion prevented the machine from repeating an EPI scan on the same m/z value after collecting data twice for that
mass value for the following 4 minutes. Peak lists were generated using a script accompanying the Analyst 1.4.2 (AB Sciex) software used to control the mass spectrometer.

**Table 4.** Parameters used to determine collision energy for EPI scans.

<table>
<thead>
<tr>
<th>Charge state</th>
<th>Slope&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Intercept&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>0.028</td>
<td>17.00</td>
</tr>
<tr>
<td>+2</td>
<td>0.028</td>
<td>17.00</td>
</tr>
<tr>
<td>+3</td>
<td>0.018</td>
<td>24.00</td>
</tr>
<tr>
<td>+4</td>
<td>0.018</td>
<td>24.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>Collision energy (CE) = Slope (m/z) + Intercept. Maximum allowable CE is 81 V.

**IMAC and HILIC.** IMAC and HILIC were performed essentially as described by McNulty and Annan [210]. For IMAC, 20 μL of a 50% (v/v) slurry of PHOS-Select iron affinity gel (Sigma) was added directly to tryptic digests of unmodified and poly(ADP-ribosyl)ated PARP-1 and vortexed for 30 min. Fractions were then placed in a 0.22 μm nylon Spin-X centrifuge tube filter (Corning) and centrifuged at 8200 x g for 30 sec. The flow-through was discarded and the gel was washed with 500 μL of 250 mM acetic acid containing 30% (v/v) acetonitrile followed by 500 μL of water. Bound material was then eluted by vortexing with 100 μL of 400 mM ammonium hydroxide for 10 min. The eluant was collected and lyophilized to dryness. Lyophilized samples were resuspended in 100 μL of 0.2% (v/v) TFA prior to Q-TRAP LC-MS analysis.

HILIC separations were performed on a Gilson preparative HPLC using a 4.6 × 250-mm TSKgel Amide-80 5-μm particle column (Tosoh Biosciences). Tryptic digest peptides were loaded in 90% solvent B (98% v/v acetonitrile with 0.1% v/v TFA) and eluted with a gradient to solvent A (98% v/v water with 0.1% v/v TFA) as follows:

1. Linear gradient to 85% solvent B over 5 min;

2. Linear gradient to 70% solvent B over 40 min; and

3. Linear gradient to 0% solvent B over 5 min.
Eighty 250 μL fractions were collected and submitted to negative mode MALDI-TOF analysis as described above for high-power MALDI MS analyses.

**General molecular biology techniques.** PCR, agarose gel electrophoresis, DNA ligation, and preparation and transformation of chemically competent cells were performed using standard procedures [211] unless otherwise stated. PCR products were purified from a slice of agarose following agarose gel electrophoresis using Qiagen's QIAquick Gel Extraction kit. Plasmid DNA was isolated using Qiagen's QIAprep Spin Miniprep Kit.

**Bacterial growth media and conditions.** Bacterial cultures were grown in LB broth with aeration at 37° C. For colony growth, cells were spread on LB-agar plates and incubated overnight at 37° C. Ampicillin (100 μg/mL), kanamycin (50 μg/mL), or chloramphenicol (34 μg/mL) was included for selection as appropriate.

**Construction of PARP-1 expression vector using Invitrogen's Gateway system.** Plasmid pCMV6-XL5 containing a PARP-1 cDNA insert was purchased from OriGene and used to transform TOP10 chemically competent cells (Invitrogen). Cells were grown overnight in LB broth with ampicillin after which transformants were selected on LB-agar ampicillin plates. An individual colony was picked and grown overnight in fresh selective LB broth. Plasmid DNA was purified and used as a template for PCR with primers containing Gateway-compatible attB sequences.

All Gateway reactions were carried out according to protocols supplied by Invitrogen. Briefly, attB-PCR products were purified and cloned into pDONR to create the PARP-1 Gateway entry clone. The PARP-1 expression clone was then created by LR recombination between the Gateway entry clone and the Gateway destination vector pDEST14 for native protein expression. This expression clone was used to transform DH5α chemically competent cells. Transformants were selected on LB-agar ampicillin plates after which individual colonies were picked and cultured overnight in selective LB broth. Plasmid DNA was purified and proper directional
insertion of the full-length PARP-1 cDNA was verified by endonuclease digestion followed by agarose gel electrophoresis.

**Synthesis of PARP-1 affinity purification resin.** The competitive PARP-1 inhibitor 3-aminobenzamide (3-AB) was coupled to ECH Sepharose 4B (GE Healthcare) via the carbodiimide method to synthesize PARP-1 affinity purification resin as follows. Twenty-five mL of ECH Sepharose 4B was washed on a sintered glass filter with 80 mL of 0.5 M NaCl per mL gel in several aliquots. The washed gel was placed in a centrifuge tube and brought to a final volume of 50 mL by addition of ddH$_2$O adjusted to pH 4.5 - 6.0 with 0.1 M sodium hydroxide. 3-AB (0.5 g dissolved in 1 mL methanol) was added and the reaction was placed on a rotator at 4° C overnight. The reaction was stopped by sequential washing with 1 L of 100 mM acetate pH 4.0 containing 0.5 M NaCl, 1 L of 100 mM Tris-HCl pH 8.0 containing 0.5 M NaCl, and finally 1 L ddH$_2$O. A slurry of 75% (v/v) settled prepared resin in 100 mM Tris-HCl pH 7.5 containing 0.02% (w/v) sodium azide was prepared and poured into a 1.5 x 20 cm glass chromatography column. The column was packed at a flow rate of 75 mL/hour and stored at 4° C.

**Overexpression and purification of PARP-1.** Chemically competent Rosetta 2 cells (Novagen) were transformed with the PARP-1 expression clone and selected on LB agar plates supplemented with ampicillin and chloramphenicol. An individual colony was picked and used to inoculate selective LB broth, then grown overnight. The culture was scaled up to 1 L and grown to an OD$_{600}$ of approximately 0.2, then allowed to cool to room temperature at which point PARP-1 overexpression was induced by addition of IPTG to a final concentration of 0.1 mM. Growth was continued over a 24-hour period after which cells were harvested by centrifugation at 6000 x g for 20 min at 4°C. Cell samples were collected at 0, 2, 4, and 24 hours after induction to assay for PARP-1 expression, which was confirmed via Western blot with anti-PARP-1 mouse mAb (Biomol).

All centrifugation steps during protein purification were performed at 4° C. The cell pellet was resuspended in 40 mL of 50 mM Tris-HCL pH 7.5 containing 250 mM NaCl and 1 mM
EDTA. Protease activity was inhibited during lysis using Roche’s Complete Mini Protease Inhibitor Cocktail Tablets according to the manufacturer’s instructions. Lysozyme was added to a final concentration of 1 μg/mL and the lysate was stirred on ice for 20 min. Triton X-100 was added to a final concentration of 0.5% (v/v) and stirring was continued for an additional 5 min. The lysate was sonicated on ice using a small probe at 25% amplitude in 1 min bursts until viscosity was reduced, and cell debris was removed by ultracentrifugation for 1 hour at 50,000 x g. DNA was removed from supernatant by precipitation with 1 mg/mL protamine sulfate followed by centrifugation for 20 min at 50,000 x g.

The supernatant was transferred to a beaker on ice and 4.585 g solid ammonium sulfate was slowly added with constant stirring for a final concentration of 35% (w/v). Stirring was continued for an additional 30 min after which the precipitate was pelleted by centrifugation for 20 min at 20,000 x g. The supernatant was returned to the beaker and 4.37 g solid ammonium sulfate was added for a final concentration of 65% (w/v) followed by stirring and centrifugation as above. The pellet, containing PARP-1, was retained.

In a cold room, the previously prepared PARP-1 affinity purification column was equilibrated with 4 bed volumes (200 mL) of binding buffer (BB) containing Tris-HCl pH 7.4 (100 mM), β-mercaptoethanol (14 mM), EDTA (0.5 mM), and PMSF (0.5 mM). The ammonium sulfate-precipitated pellet containing PARP-1 was resuspended in 150 mL BB buffer and loaded onto the column overnight at a flow rate of 10 mL/hour. The column was washed successively with three 200 mL aliquots of BB buffer of increasing ionic strength (100 mM NaCl, 400 mM NaCl, and 800 mM NaCl). The flow rate was increased to 50 mL/hour and PARP-1 was eluted with 200 mL BB buffer containing 3-methoxybenzamide (1 mM) and 400 mM NaCl. Aliquots (8 x 10 mL) were collected and protein concentrations were determined by the Bradford method [209] with a BSA standard using Pierce’s Coomassie Plus reagent kit. The protein-containing fraction was concentrated ten-fold using a 10 kDa MWCO concentrator (Vivaspin Hydrosart) and stored at -80° C.
Results and Discussion

Ammonium sulfate fractionation can be used to enrich for covalently poly(ADP-ribosyl)ated proteins. Proteins were salted out from U2-OS whole cell lysates in a stepwise fashion using increasing concentrations of ammonium sulfate. The resulting pellets were then rehydrated, dialyzed into PBS, and submitted to SDS-PAGE analysis. Coomassie staining indicated that the majority of protein precipitated out at 40-60% salt, a result in agreement with typical ammonium sulfate precipitation ranges [200]. However, an anti-PAR immunoblot of the same fractions indicated that the majority of PAR remained in the supernatant (Figure 5A). While it is generally true that only low-molecular weight proteins remain in solution at ammonium sulfate concentrations of 80% and above, most of the PAR detected was of relatively high molecular weight. It therefore seemed likely that the bulk of PAR found in the supernatant was not covalently bound to protein and that noncovalent protein-PAR interactions had been interrupted by the extremely high ionic strength.

However, the possibility still existed that covalent poly(ADP-ribosyl)ation greatly increases a protein’s solubility such that it remains in solution despite high salt. To rule out this scenario, we performed an anti-PARP-1 immunoblot on salt-fractionated lysates from U2-OS cells that had been treated with H$_2$O$_2$ to activate PARP and thus stimulate PAR formation [212]. In untreated cells, PARP-1 was found strongly concentrated in the 60% fraction; however, in treated cells, a significant portion of PARP-1 shifted to the 40% fraction, indicating a substantial decrease in solubility (Figure 5B). As PARP-1 is the primary acceptor of PAR in human cells, we concluded that this solubility shift was caused by the addition and/or extension of PAR polymer chains and thus that poly(ADP-ribosyl)ation causes a decrease, not an increase, in solubility. This work supports the conclusion that while the majority of PAR in human cells is not covalently bound to protein, covalent poly(ADP-ribosyl)ation does exist, in all likelihood on PARP-1. Furthermore, it demonstrates that ammonium sulfate fractionation may be used as a tool to separate free PAR from covalently poly(ADP-ribosyl)ated proteins. Increasing the salt concentration in smaller increments could provide even finer separation of poly(ADP-ribosyl)ated
Figure 5. Ammonium sulfate fractionation is an enrichment tool for covalently poly(ADP-ribosyl)ated proteins. (A) Whole cell lysates from U2-OS cells were subjected to ammonium sulfate fractionation followed by SDS-PAGE. While the majority of protein precipitates at 40-60% salt as indicated by Coomassie staining, an anti-PAR immunoblot demonstrates that most PAR remains in the supernatant and is probably not covalently associated with protein. (B) Ammonium sulfate fractionation and Western blot analysis of whole cell lysates from U2-OS cells treated with hydrogen peroxide to activate PARP-1. Upon oxidative damage, higher molecular weight PARP-1 shifts from the 60% to the 40% salt fraction, indicating a substantial decrease in solubility likely caused by covalent addition and/or extension of pADPr polymer chains.
protein according to PAR chain length. This approach would substantially reduce sample complexity and could prove invaluable to mass spectrometric analysis of poly(ADP-ribosyl)ation in whole cell lysates in the future.

**Boronate affinity resin retained poly(ADP-ribosyl)ated protein, but bound material could not be recovered.** We then turned to evaluation of boronate affinity chromatography to enrich for poly(ADP-ribosyl)ated protein. Recombinant PARP-1 was incubated with a reaction buffer containing NAD, then subjected to boronate affinity chromatography in a mini spin column format. Volatile buffers were used in order to simplify possible future application of the technique to MS sample preparation; the binding buffer was ammonium bicarbonate pH 8.0, and the elution step was accomplished by washing with ammonium acetate pH 6.0. Guanidine-HCl was included in both the wash and elution buffers to prevent nonspecific protein binding to the resin [213]. Flow-throughs and elutions from the column were then analyzed by Western blot, which indicated that PARP-1 was found only in the flow-through. To determine whether protein was still bound to the resin, the experiment was repeated and material from the boronate beads was released by boiling in Laemmli SDS-PAGE sample buffer containing β-mercaptoethanol. As shown in Figure 6, significant PARP-1 was indeed found in the bead fraction. Subsequent experiments using an elution buffer of lower pH as well as a low-pH elution buffer containing sorbitol produced virtually identical results (data not shown), indicating that the extremely strong affinity of poly(ADP-ribosyl)ated material for the beads may not have strictly been a function of the boronate/cis-diol interaction.

While boronate/cis-diol ester formation is the main mechanism of boronate affinity chromatography, the full scope of the interactions involved is not fully understood. Dissociation constants for phenylboronic acid diesters are much higher than is typical for ligands used in affinity chromatography, leading to speculation that secondary interactions such as hydrophobic interactions, ionic interactions, and hydrogen bonding are also at work [203]. Properties of PARP-1’s primary, secondary, and/or tertiary structure may be exacerbating the effects
Boronate affinity resin retains poly(ADP-ribosyl)ated PARP-1. Recombinant PARP-1 was incubated with a reaction buffer containing NAD, then subjected to boronate affinity chromatography followed by Western blot analysis. The material that remained bound to the affinity resin after two low-pH elutions was released by boiling in Laemmli SDS-PAGE sample buffer containing β-mercaptoethanol. The majority of PARP-1 was found in the flow-through (FT). (The low MW band seen in these lanes is a degradation product that was present in recombinant PARP-1 after purification from E. coli.) PARP-1 was evident in neither the first nor second elution (EL 1 and EL 2). The remainder of the PARP-1 remained bound to the beads. The higher apparent MW of these bands (150 kD as opposed to 113 kD for unmodified PARP-1) indicates that this material is poly(ADP-ribosyl)ated. The presence of some bound material in the control lane indicates that the purified recombinant purified PARP-1 was already poly(ADP-ribosyl)ated to some extent.

Figure 6.

of these secondary interactions, impeding the recovery of bound material. Furthermore, all previous studies involving boronate affinity chromatography of ADP-ribose were performed on free PAR [202, 214, 215] or mono-ADP-ribosylated histone [213]. The behavior of material containing longer PAR oligomers attached to a large protein — PARP-1 is 113 kD whereas histone varieties generally fall between 11-21 kD — was therefore unknown prior to this work. Boronate affinity chromatography may still prove useful in sample enrichment for poly(ADP-ribosyl)ated protein; however, for our purposes a reliable means of dissociating bound material from the affinity resin without the addition of compounds that would interfere with MS analysis would first have to be determined. The possibility of using boronate affinity chromatography to separate poly(ADP-ribosyl)ated tryptic peptides rather than whole proteins should also be explored.

Poly(ADP-ribosyl)ated histone H1 could not be detected by LC-MS/MS in a solid-state system. To test the efficacy of a solid-state system in reducing sample complexity after poly(ADP-ribosyl)ation prior to MS analysis, histone H1 was immobilized to a 96-well plate and subjected to poly(ADP-ribosyl)ation by the addition of a reaction buffer containing PARP-1 and biotinylated NAD. The supernatants, containing automodified PARP-1 and free PAR, were
then removed and analyzed separately from the well-bound material, containing poly(ADP-ribosylated) histone. Using colorimetric detection and Western blotting, it was determined that PAR was present in both the well-bound material and the reaction supernatants following the initiation of PARP-1 activity (Figure 7A and B). However, because PAR has been shown to participate in non-covalent interactions with histones [196, 197], it was not known whether the well-bound PAR was covalently attached to protein.

To look for evidence of covalent histone modification, the well-bound material was subjected to tryptic digest followed by LC-MS/MS analysis. We were particularly interested in the modification status of the N-terminal acetyl peptide, as this “tail” region is known to associate with PAR [196, 197] and contains two glutamate residues. A mass table of the N-terminal peptide along with the MS/MS spectrum is shown in 8. No ions with a mass corresponding to ADP-ribosyl cleavage were detected, nor were ions that had similar mass spectral fragmentation patterns. These results likely indicated either that extended PAR chains had interfered with fragment ionization, or that the well-bound PAR had been noncovalently associated with histone.

Figure 7. PAR is present in both the well-bound material and the supernatants following initiation of PARP-1 activity in a solid-state system. Histone H1 was immobilized to 96-well plates via passive adsorption and incubated with a PARP-1 reaction mixture containing biotinylated NAD. The presence of well-bound PAR was verified via streptavidin-HRP detection and color development with TACS-Sapphire (Trevigen), a chromagen which absorbs at 460 nm after acidification (A). The presence of PAR in the reaction supernatants was verified via anti-PAR Western blot. Error bars represent standard deviation.
MS analysis also revealed that PARP-1 was found in the well-bound material (data not shown), demonstrating that the solid-state setup does not in fact successfully separate from auto-poly(ADP-ribosyl)ation as was asserted by Kirsten et al. [204]. We hypothesized that noncovalent interactions between some combination of histone, PAR, and PARP-1 had caused association of PARP-1 with the plate. It was also possible that PARP-1 was binding nonspecifically to the the plastic. While any nonspecific adsorption may have been preventable with application of a blocking agent prior to addition of the PARP reaction mixture, such a step would have complicated the analysis by introducing another protein or set of proteins to the system. In any event, due to these findings the decision was made to discontinue the use of solid-state poly(ADP-ribosyl)ation as a means of reducing sample complexity.

Figure 8. No N-terminal histone modifications were detected by LC-MS/MS after in vitro poly(ADP-ribosyl)ation using a solid-state system. Well-bound material was subjected to tryptic digest followed by LC-MS/MS analysis. The N-terminal acetyl peptide was retained and re-fragmented as this region of histone H1 is known to associate with PAR [197]. (A) A mass table of expected fragments from the N-terminal acetyl peptide was generated using Mascot. Fragments detected in this experiment are indicated in red. No poly(ADP-ribosyl)ated residues were found. (B) MS2 spectrum of the N-terminal acetyl peptide showed no unusual modifications.

Free poly(ADP-ribose) can be analyzed by negative mode MALDI-TOF mass spectrometry. As no evidence of poly(ADP-ribosyl)ation was found during our initial attempts at positive
mode LC-MS/MS, we decided to simplify our approach substantially by attempting MALDI-TOF MS of free PAR polymer. It was hypothesized that removing protein from the system would greatly increase chances of success by permitting negative mode analysis; PAR is more likely to be detected in negative mode, but interpretation of negative mode protein spectra is quite difficult [199]. Analysis was performed on a commercial preparation of PAR (Biomol) containing polymers ranging from 2-300 units in length with an average length of 20 units. A representative spectrum demonstrating the detection of oligomers from 6-11 units in length is given in Figure 9. As illustrated in Table 5, the majority of the oligomers detected in the experiment had undergone a loss of the terminal ribose-5-phosphate. (It is not yet known whether this loss is a result of in-source decay; this will require further study.) Moreover, no oligomers longer than 11 units were detected despite the fact that the analyzed mixture contained a wide range of polymer chain lengths. This confirms that PAR polymers do not ionize well in the gas phase, suggesting that a peptide or protein modified by a long PAR chain is unlikely to be detected during MS analysis.

It should also be noted that this is to our knowledge the first MALDI-TOF analysis of poly(ADP-ribose). While it has been shown that cell growth conditions can affect both the length and branching density of pADPR produced in vivo, these relationships are not understood. Leaders in the field have therefore identified the investigation of PAR structural variations and the putative “poly(ADP-ribose) code” as important areas of research [47]. MALDI-TOF presents an option for PAR analysis that does not involve the use of radioactive labels, unlike other approaches currently in use.

**SVPDE treatment alters the MS spectra of both unmodified and poly(ADP-ribosyl)ated protein.** As it was becoming increasingly clear that any successful identification of poly(ADP-ribosyl)ation by mass spectrometry would involve reduction of polymer chain length, we then sought to test the efficacy of SVPDE digestion for this purpose. Histone H1 was incubated with a reaction buffer containing PARP-1 and NAD. Reactions were terminated by TCA precipita-
**Figure 9.** *Free poly(ADP-ribose) can be analyzed by negative mode MALDI-TOF mass spectrometry.* A commercial preparation of PAR was analyzed by MALDI-TOF in negative mode and data was collected for m/z of 200-8000. A representative spectrum demonstrating the detection of various PAR oligomer derivatives from 6-11 units in length is shown. The structure of a PAR trimer with the terminal AMP tail and ribose-5-phosphate highlighted is given for reference.
Table 5. Mass table of poly(ADP-ribose) oligomers and derivatives. Species detected in this experiment are indicated in red. The majority of detected oligomers had undergone loss of the terminal ribose-5-phosphate. No oligomers longer than 11 units were detected.

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tion, resuspended, and incubated in the presence of SVPDE, then subjected to whole protein MALDI-TOF analysis in positive mode. The resulting control histone spectrum featured two sets of characteristic triplet peaks representing the +1 and +2 ions of three H1 isomers (Figure 10A). Histone H1 incubated with PARP-1 alone produced a spectrum virtually identical to the control (Figure 10A and B); this was attributed to the suppression of signal from any modified histone by long PAR chains. However, SVPDE treatment altered the MS spectra of histone regardless of poly(ADP-ribosyl)ation status (Figure 10C and D). The signal intensity of the characteristic triplet peaks was greatly reduced, and two new sets of peaks appeared around m/z ratios of approximately 13 and 15 k. These peaks were of unknown origin and could not be readily attributed to SVPDE itself, as the enzyme’s molecular weight is 115 kDa. It is possible that the commercial SVPDE preparation used in the experiment contained some contaminant(s) capable of altering the spectra. In any event, because SVPDE digestion did not appear to enable MS identification of poly(ADP-ribosyl)ated protein, the decision was made to explore other means of reducing PAR chain length.
Furthermore, several difficulties arising from the use of histones as a poly(ADP-ribosylation) target arose in the process of data analysis for this experiment. First, it became apparent that histone material in solution is capable of spontaneous aggregation over time, as higher molecular weight peaks began to appear in the spectra as the samples aged (data not shown). Secondly, the existence of numerous histone H1 isomers with different primary sequences complicated the system and rendered manual inspection of MS/MS fragmentation data exceedingly difficult. This was a major roadblock; considering that poly(ADP-ribosyl)ated peptides will not be assigned to protein hits in a Mascot search, the ability to successfully analyze fragmentation data “by hand” to some extent is important. The focus of subsequent experiments was therefore shifted to automodification of PARP-1.

**LC-MS/MS analysis of automodified PARP-1 identified potential poly(ADP-ribosylation) targets.** The results from MALDI-TOF analysis of free PAR polymer led us to hypothesize that a comparison of the LC-MS spectra of unmodified vs. automodified PARP-1 could yield insight to potential poly(ADP-ribosylation) targets, as peptides containing these residues would seem to disappear upon poly(ADP-ribosylation). To explore this idea, PARP-1 was incubated with a reaction buffer containing NAD and subjected to tryptic digest followed by LC-MS/MS analysis. Indeed, the signal intensities of two particular +2 ions with m/z ratios of 681.8 (Figure 11A) and 958.4 (Figure 11B) were shown to decrease dramatically in poly(ADP-ribosyl)ated PARP-1. MS/MS identified the corresponding protein sequences as $^{552}$VFSATLGLVDIVK$^{564}$ and $^{638}$FYPLEIDYGQDEEAVK$^{653}$, respectively; this was confirmed by manual inspection of the fragmentation data. Although $^{552}$VFSATLGLVDIVK$^{564}$ contains no glutamate residues and $^{638}$FYPLEIDYGQDEEAVK$^{653}$ lies outside the classical PARP-1 automodification domain, recent studies demonstrate that in reality, poly(ADP-ribosylation) of PARP-1 is not confined to glutamate residues or the AD as had been assumed for many years [216, 217]. We therefore consider both of these peptides to be potential poly(ADP-ribosylation) targets. While it is not a “smoking gun,” the disappearance of a peptide after poly(ADP-ribosylation) may be indicative of modifi-
Figure 10. SVPDE treatment alters the MS spectra of both unmodified and poly(ADP-ribosyl)ated protein. Histone H1 was incubated with a reaction buffer containing PARP-1 and NAD. Reactions were terminated by TCA precipitation, resuspended, and incubated in the presence of SVPDE, then subjected to whole protein MALDI-TOF analysis in positive mode; data was collected for m/z of 5000-35000. (A) The control spectrum features two characteristic triplet peaks representing the +1 and +2 ions of three H1 isomers. (B) Histone H1 incubated with PARP-1 alone produced a spectrum virtually identical to the control; this was attributed to the suppression of signal from any modified histone by long PAR chains. (C and D) SVPDE treatment altered the MS spectra of histone regardless of poly(ADP-ribosyl)ation status. The signal intensity of the characteristic triplet peaks was greatly reduced, and two new sets of peaks of unknown origin appeared around m/z ratios of approximately 13 and 15 k.
cation and could be used to identify potential targets for further investigation.

**LC-MS/MS analysis of free PAR polymer generates several "signature ions."** Because poly(ADP-ribosyl)ation appears to significantly decrease the signal intensity of modified peptides, we sought to determine whether fragmentation of free PAR would produce characteristic “signature ions.” By scanning for the release of these ions during MS/MS, one could potentially pinpoint (and identify via MS$^2$ spectra analysis) the peptides from which they originated. A commercial preparation of PAR was subjected to LC-MS/MS on a Q-TRAP mass spectrometer operating in negative mode. As shown in Figure 12, fragmentation during MS analysis resulted in the release of several signature ions, including phosphate, cAMP, and AMP. (The possibility that these ions originated from contaminants in the commercial PAR preparation rather than from the polymer itself could not be discounted from these results alone; however, their origin was verified in subsequent experiments using *in vitro* automodified PARP-1, as will be discussed below.) The signature ions identified in this work could be used to perform precursor ion scanning, a mass spectrometric technique for the indentification of ions giving rise to specific products. In precursor ion scanning with a Q-TRAP mass spectrometer, the second quadrupole is set to transmit only ions of a particular m/z while the first mass filter scans the mass range including ions whose fragmentation would result in the selected product ion. [218]. This significantly enhances resolution and sensitivity [219], which could help overcome the effects of PAR-mediated signal suppression.

**High-power MALDI-TOF/TOF analysis of short-chain automodified PARP-1 enables detection of signature ions.** Despite our determination that free PAR releases signature ions upon fragmentation, initial attempts to replicate this result with PARP-1 automodified *in vitro* were unsuccessful (data not shown). Once again, we hypothesized that extended PAR chains were interfering with the ionization process. While it is true that long PAR chains were present in the commercial preparation used in the previous experiment, it is quite plausible that the polymer would behave differently when attached to protein. Several reports have demonstrated
that the length of PAR polymers synthesized during \textit{in vitro} poly(ADP-ribosyl)ation is dependent upon substrate concentration; if the NAD concentration is kept in the nanomolar rather than micromolar range, oligomers tend to be quite short — typically, from 1 to 3 ADP-ribose units [205, 206]. We chose to take advantage of this property of the enzyme rather than adding extraneous proteins to the system for polymer degradation. This approach was combined with a high-power MALDI technique in which the laser intensity was set higher than normal to increase the chances that large molecules would fragment during ionization. PARP-1 was incubated with a reaction buffer containing either nanomolar or millimolar NAD, then subjected to tryptic digest followed by high-power MALDI-TOF/TOF analysis in negative mode. As shown in Figure 13, levels of PAR signature ions detected from the nM NAD reaction were increased 14- to 27-fold over those of the control reaction. As these samples were subjected to buffer exchange prior to MS analysis, it can be concluded that the observed signature ions originated from PAR polymer and not from small molecule contaminants. Levels of these ions released from the micromolar NAD reaction were slightly increased over the control but still quite low, further demonstrating that the observed peaks did not originate from residual free NAD. These results indicate that management of polymer length is important not only to the detection of ions originating from poly(ADP-ribosyl)ated peptides, but to the detection of ions resulting from fragmentation of protein-associated PAR as well. This point is critical to the potential success of precursor ion scanning in MS analysis of poly(ADP-ribosyl)ation, and could explain why such approaches have proven fruitful only in the analysis of mono-ADP-ribosylation thus far [220].

**IMAC identified a potential poly(ADP-ribosyl)ation target from the PARP-1 automodification domain.** Finally, we evaluated the use of immobilized metal affinity chromatography (IMAC) for the separation of poly(ADP-ribosyl)ated peptides prior to MS analysis. PARP-1 was incubated with a reaction buffer containing NAD and subjected to tryptic digest followed by IMAC. Eluted material from the IMAC beads was then analyzed by LC-MS/MS. No peptides were identified in the IMAC-bound material from unmodified PARP-1; however, one peptide
from poly(ADP-ribosyl)ated PARP-1 was found associated with the IMAC beads. MS/MS analysis identified the sequence as 487AEPVEVVAPR496 and this result was confirmed by manual inspection of the MS/MS spectrum (Figure 14).

Interestingly, this is one of two peptides from the PARP-1 automodification domain that were determined to be poly(ADP-ribosyl)ated in a concurrent study by another research group [217]. We therefore feel reasonably confident that the isolated peptide was in fact poly(ADP-ribosyl)ated. However, attempts to replicate the result were unsuccessful. As the initial study had been performed with long-chain modified PARP-1 and we now know that extended PAR chains interfere with ion detection, we repeated the experiment using PARP-1 automodified in vitro with nanomolar rather than micromolar NAD. No peptides were found associated with the IMAC beads despite multiple attempts. We hypothesize that while IMAC resin designed for purification of phosphopeptides does have some affinity for PAR, long polymer chains are required for successful binding. This introduces a conundrum: long-chain poly(ADP-ribosyl)ation allows peptides to be purified by IMAC, but subsequently tends to prevent successful identification of those peptides by MS/MS. We speculate that an analyzable MS$^2$ spectrum was obtained for poly(ADP-ribosyl)ated 487AEPVEVVAPR496 only because the peptide happened to lose its attached PAR chain during the ionization process.

Nevertheless, this work demonstrates for the first time that IMAC has promise as a method for the separation of poly(ADP-ribosyl)ated protein. An approach can be envisioned in which long-chain poly(ADP-ribosylated) protein is subjected to tryptic digest and bound to IMAC resin, followed by polymer digestion (e.g. with PARG) or base-catalyzed cleavage of the PAR-protein linkage [215] to allow successful MS/MS identification of the modified peptides. Furthermore, a recent study from McNulty et al. has demonstrated increased utility of IMAC when used in conjunction with hydrophilic interaction chromatography, or HILIC, a high resolution separation technique in which hydrogen bonding between a peptide and the hydrophilic stationary phase provides the primary interaction, separating peptides on the basis of hydrophilicity. Performing HILIC prior to IMAC separation has been shown to greatly enhance
sensitivity and selectivity in the identification of peptides containing hydrophilic modifications such as phosphorylation [210]. It is therefore reasonable to speculate that the HILIC/IMAC method would similarly improve separation and detection of poly(ADP-ribosyl)ated peptides, as the PAR polymer chain is hydrophilic due to its phosphate moieties. In separate experiments, we have demonstrated that negative mode MALDI analysis of HILIC fractions from a tryptic digest of poly(ADP-ribosyl)ated histone H1 identifies two fractions which release PAR signature ions upon fragmentation (Figure 15). However, interpretation of the MS/MS spectra from those fractions was difficult due to the existence of multiple histone isomers with different primary sequences, as discussed above. We believe that simplifying the system by instead applying HILIC/IMAC to automodified PARP-1 is likely to prove a fruitful approach to the mass spectrometric identification of poly(ADP-ribosyl)ation sites.
Figure 11. The signal intensity of ions originating from two PARP-1 tryptic peptides is greatly reduced upon poly(ADP-ribosyl)ation. Analysis of an in vitro PARP-1 automodification reaction by LC-MS: a linear ion trap enhanced mass spectrum scan in positive ion mode was first collected for m/z from 400-2400, and ions of high intensity were then analyzed by enhanced product scan for m/z from 225-1515. Averaged enhanced product scans from two separate runs are shown; spectra from unmodified PARP-1 (black) are overlaid with the corresponding spectra from poly(ADP-ribosyl)ated PARP-1 (red). The signal intensities of two +2 ions ions with m/z ratios of 681.8 (A) and 958 (B) were greatly reduced upon poly(ADP-ribosyl)ation. MS/MS analysis confirmed that these ions originated from \textit{VFSATLGLVDIVK} and \textit{FYPLEIDYGQDEEAVK}, respectively. (Ions in the 938-947 mass range of spectrum B presented as broad peaks due to several overlapping peptides with similar molecular weights.)
Figure 12. **LC-MS/MS analysis of free pADPr generates several signature ions.** A commercial preparation of PAR containing polymers ranging from 2-300 units in length with an average length of 20 units was subjected to LC-MS/MS analysis on a Q-TRAP mass spectrometer operating in negative mode; ions in the m/z range of 50-1800 were collected and refragmented. PAR was found to release phosphate (Mr = 97.991), cAMP (Mr = 329.206), and AMP (Mr = 347.22).

Figure 13. **High-power MALDI-TOF analysis of short-chain poly(ADP-ribosyl)ated peptides releases signature ions.** PARP-1 was incubated with either nanomolar or micromolar NAD, then subjected to tryptic digest followed by MALDI-TOF/TOF analysis in negative mode. Data was collected over m/z of 200-2000. The laser intensity was set higher than normal in order to encourage fragmentation of large molecules. Levels of PAR signature ions released from the nM NAD reaction were strikingly increased over those of both the control and the micromolar NAD reaction.
**Figure 14.** Identification of a PARP-1 tryptic peptide isolated after IMAC was confirmed by LC-MS/MS. PARP-1 was incubated with a reaction buffer containing NAD and subjected to tryptic digest followed by IMAC separation. Eluted material from the IMAC beads was analyzed by LC-MS/MS. A peptide from the PARP-1 automodification domain was identified from the IMAC-bound poly(ADP-ribosylated material. Its sequence was confirmed as **487**AEPVEVVAPR**496** by MS² analysis. (A) Mass table of expected ions and (B) MS² spectrum of **487**AEPVEVVAPR**496**. Ions detected in this experiment are highlighted in red.

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</tbody>
</table>

**Figure 15.** MALDI-TOF/TOF analysis of HILIC fractions from a tryptic digest of poly(ADP-ribosyl)ated histone H1 identified two fractions releasing PAR signature ions. A tryptic digest of poly(ADP-ribosyl)ated histone H1 was subjected to HILIC fractionation followed by MALDI-TOF/TOF MS to scan for the release of PAR signature ions including ADP-ribose (blue) as well as two additional ions identified by Morrison *et al.* (purple and green) [190]. Of note are fractions 20 and 32 which show peaks indicating the presence of PAR. The early peaks (fractions 1-6) likely originate from free ADP-ribose and/or short PAR oligomers, while the very late peak (fraction 70) appears to arise from free NAD.
**Conclusion**

The objective of this study was to develop viable approaches for the analysis of *in vitro* poly(ADP-ribosyl)ated proteins by mass spectrometry. Though we had originally hoped to establish methods for *in vivo* work as well, it soon became apparent that the problem was much too complex to permit this. Nevertheless, we were able to evaluate and in some cases refine a number of different biochemical and mass spectrometric techniques which show promise for future studies. Results, conclusions, and next steps for all tested methods are summarized in Table 6.

While this work provided several insights that will help pave the way for successful mass spectrometric analysis of poly(ADP-ribosyl)ation, a major stumbling block arose from the fact that protein-bound PAR appears to fragment in unexpected ways during ionization in the gas phase. Based in part upon the results from our MALDI-TOF analysis of free PAR, we had predicted that the polymer would fragment primarily along certain labile bonds in its backbone (Figure 4) and used this assumption to generate a theoretical mass table of poly(ADP-ribosyl)ated PARP-1 tryptic peptides to aid in the analysis of MS\(^2\) spectra. However, a recent study by Hengel *et al.* indicates that ADP-ribose commonly fragments at a number of other points which we did not anticipate (Figure 16). This rendered our mass tables incomplete and decreased our chances of identifying poly(ADP-ribosyl)ated peptides. Corrected tables will be utilized in future studies. Still, this new information about ADP-ribose fragmentation behavior demonstrates that MS analysis of ADP-ribose oligomers is even more complex than originally thought.

The findings of Hengel *et al.* also emphasize the critical importance of reducing sample complexity. In that work, kemptide, a short synthetic peptide (amino acid sequence LRRASLG), was mono-ADP-ribosylated *in vitro* and subjected to LC-MS/MS analysis. Even using a system of maximum simplicity, identification of the modification site was not trivial; ADP-ribosylated kemptide produced none of the expected *b* or *y* ions associated with the unmodified peptide [221], a finding in agreement with our observation that poly(ADP-ribosyl)ated peptides seem to
**Table 6. Summary of results and conclusions/next steps for various sample preparation approaches for MS analysis of poly(ADP-ribosylation).**

<table>
<thead>
<tr>
<th>Approach</th>
<th>Results</th>
<th>Conclusions/Next steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>Successfully separated free PAR from poly(ADP-ribosyl)ated protein</td>
<td>Will be of great utility in reducing sample complexity for future MS analyses of <em>in vitro</em> poly(ADP-ribosyl)ation of proteins</td>
</tr>
<tr>
<td>Boronate affinity chromatography</td>
<td>Successfully isolated poly(ADP-ribosyl)ated protein; however, bound material could not be recovered for MS analysis</td>
<td>Possibly useful, but must first establish methods for dissociating poly(ADP-ribosyl)ated protein from boronate affinity resin without introduction of compounds that interfere with MS analysis</td>
</tr>
<tr>
<td>Solid-state poly(ADP-ribosylation)</td>
<td>Did not allow easy physical separation of trans- and auto-poly(ADP-ribosyl)ated protein</td>
<td>Not a good choice for reducing sample complexity prior to MS analysis of poly(ADP-ribosyl)ated protein</td>
</tr>
<tr>
<td>SVPDE digestion</td>
<td>Altered the MS spectra of proteins independent of poly(ADP-ribosyl)lation status</td>
<td>Not a good choice for reducing chain length and sample complexity prior to MS analysis of poly(ADP-ribosyl)ated protein</td>
</tr>
<tr>
<td>Poly(ADP-ribosylation) with nM NAD</td>
<td>Successfully reduced PAR chain length to allow MS detection of signature ions from poly(ADP-ribosyl)ated protein</td>
<td>Will be of great utility for MS analysis of poly(ADP-ribosyl)ation by wild-type PARP-1</td>
</tr>
<tr>
<td>IMAC</td>
<td>Successfully isolated a possible poly(ADP-ribosyl)ated peptide from the PARP-1 automodification domain</td>
<td>Likely useful, especially in conjunction with HILIC, but long-chain poly(ADP-ribosyl)ation appears to be required for protein binding; must first establish methods for reducing PAR chain length after IMAC and prior to MS analysis</td>
</tr>
</tbody>
</table>
Figure 16. Empirically determined ADP-ribose fragmentation pattern from arginine-linked mono-ADP-ribosylated kemptide. ADP-ribosylated kemptide (peptide sequence LRRASLG) was subjected to LC-MS/MS analysis. Identified fragment ions containing the intact peptide backbone plus a portion of the modification are labeled p_1 through p_{10}. Identified fragment ions independent of the peptide sequence, only containing the ADP-ribose modification, are labeled m_1 through m_{10}. From Hengel, S. M. et al. Tandem Mass Spectrometry Investigation of ADP-ribosylated Kemptide. *J Am Soc Mass Spectrom* **20**, 477-483 (2009). ©2009, Elsevier. Reprinted with permission.
“disappear” from LC-MS/MS mass spectra. Considering the relative complexity of the samples analyzed in our work, it is unsurprising that initial attempts did not yield specific identification of poly(ADP-ribosyl)ation sites. One might expect that the first successful MS analysis of poly(ADP-ribosyl)ation by PARP-1 would involve a more reductive system.

Indeed, Tao et al. published a report identifying three poly(ADP-ribosyl)ation sites in the PARP-1 automodification domain [217] as the work described in this chapter was nearing conclusion. The Tao et al. study did not evaluate wild-type PARP-1; instead, it employed an E988Q mutant which catalyzes mono- rather than poly(ADP-ribosyl)ation [222, 223]. This mutant was used to modify a His$_6$-tagged construct of the PARP-1 automodification domain corresponding to residues 374-525 (referred to as domain D). The system was therefore highly simplified with regard to both the length of the ADP-ribose modification and the peptide mixture to be analyzed. When mono-ADP-ribosylated domain D was subjected to tryptic digest followed by LC-MS/MS, two ADP-ribosylated tryptic peptides were identified — one being AEPVEVVAPR, the same peptide isolated in our IMAC experiment — and the modified amino acid residues were determined to be D387, E488, and E491 by examination of the MS$^2$ spectra. Interestingly, ADP-ribosylation was reduced but not completely abrogated in aspartate/glutamate -> alanine mutants of domain D for these three residues. Furthermore, ADP-ribosylation of an E988Q triple mutant was similar to that of E988Q. These findings indicate that not only are there are other undiscovered ADP-ribose acceptor sites within the classical PARP-1 automodification domain, but the “automodification domain” may not even be the primary region of PARP-1 poly(ADP-ribosyl)ation after all. This idea is corroborated by a report from Altmeyer et al. which used amino acid substitutions to demonstrate that lysine residues both within and outside the classical PARP-1 automodification domain appeared to be the dominant acceptors of poly(ADP-ribose) in full-length PARP-1 [216]. Taken together, these studies disprove basic assumptions about the nature of PARP-1 which have been widely held for almost 20 years. It should also be noted that Tao et al. observed frequent dissociation of ADP-ribose from modified peptides during ionization, indicating that the ester linkage between ADP-ribose and the
acceptor residue is labile and suggesting that enrichment for ADP-ribosylated protein prior to MS analysis is likely a critical step.

Based upon our observations from the work outlined in this chapter in combination with the takeaway points from the Hengel and Tao studies, we have designed a composite scheme for the identification of poly(ADP-ribosyl)ated peptides from full-length wild-type PARP-1 by mass spectrometry (Figure 17). The workflow begins with purified recombinant PARP-1 subjected to both short- and long-chain poly(ADP-ribosyl)ation followed by tryptic digest. It then splits into three different branches designed to generate an overlapping list of peptides exhibiting evidence of poly(ADP-ribosyl)ation. In the event that specific modified residues cannot be discerned by analysis of MS\(^2\) spectra from the tryptic digest of full-length PARP-1, this information could be verified by synthesis of the peptide(s) of interest followed by LC-MS/MS. The fact that PAR and its signature ions are more easily detected in negative mode while peptide identification and sequencing is typically performed in positive mode has been a confounding issue. Our proposed scheme addresses this complication by first calling for negative mode analyses to identify HILIC fractions or LC-MALDI spots releasing PAR signature ions, followed by positive mode analyses of the identified fractions/spots to provide peptide sequence information.

Though several PARP-1 automodification target residues have now been established, there is still much to be done. First, it is clear that the three residues identified by Tao et al. do not comprise the extent of PARP-1 automodification sites. But moreover, in order to fully understand how cellular processes are regulated by poly(ADP-ribosyl)ation, we must be able to analyze proteins poly(ADP-ribosyl)ated \textit{in vivo} and can therefore not rely on techniques requiring the use of truncated and/or mutated PARP-1. In this work, we have identified several promising methods for the mass spectrometric analysis of full-length wild-type automodified PARP-1, and outlined a strategy which, with the addition of enrichment and purification steps such as ammonium sulfate fractionation, could easily be applied to the analysis of poly(ADP-ribosyl)ated proteins from cell lysates in the future.
Figure 17. Schematic for identification of poly(ADP-ribosyl)ated peptides by mass spectrometry. We have developed a composite scheme for the identification of poly(ADP-ribosyl)ated peptides by mass spectrometry based on the successful approaches evaluated in this work. Purified recombinant PARP-1 is subjected to short- and long-chain poly(ADP-ribosyl)ation followed by tryptic digest. A portion of these digests is then analyzed by classical positive mode LC-MS/MS (red boxes) to yield a list of peptides which disappear upon poly(ADP-ribosyl)ation (red circle). The long-chain tryptic digest (yellow boxes) is subjected to HILIC and IMAC, after which the attached PAR chain is either cleaved completely with base, or digested to the final ADP-ribose by PARG. The resulting material is either analyzed by positive mode LC-MS/MS, or analyzed by negative mode LC-MALDI to identify phosphate-containing spots followed by positive mode LC-MALDI to identify peptides from those spots. This branch of the schematic yields a list of IMAC-associated peptides (yellow circle). Finally, the short-chain tryptic digest (purple boxes) is subjected to HILIC and negative mode MALDI-TOF/TOF to identify PAR-containing fractions, followed by positive mode Q-TRAP LC-MS/MS to identify peptides from those fractions. As ions originating from modified peptides will likely not be assigned to protein hits in a Mascot search, it may be necessary to manually match unassigned peptides to a theoretical mass table of poly(ADP-ribosyl)ated PARP-1 tryptic peptides (purple circle).
Introduction

The Cellular Senescence Model of Aging

Aging can be defined as “a time-dependent decline in physiological capacity, associated with a decreased ability to cope with environmental stresses and an increased risk of morbidity and mortality” [224]. While this definition pertains largely to aging on an organismal level, it is widely accepted that the cumulative effect of changes occurring in individual cells and groups of cells can contribute to the aging of an organism as a whole. Among the changes implicated in the aging process is the phenomenon of cellular (or replicative) senescence, an arrest of cell proliferation that is irreversible under normal conditions. Hayflick observed in 1965 that human diploid fibroblasts growing in culture undergo an initial period of rapid proliferation followed by a decrease in growth rate and an eventual cessation of replication [174]. In addition to this loss of proliferative capacity, most senescent cells display a set of characteristic phenotypes including an enlarged and flattened morphology, decreased protein synthesis and degradation, resistance to apoptosis, and increased nucleus and nucleolus size [225]. A shift in the nature of the extracellular environment, including the stability of the extracellular matrix and the char-
acter of the secreted proteome, has also been described [226]. Several decades after Hayflick's discovery it was proposed that cellular senescence occurs largely due to the progressive shortening of telomeres during DNA replication [227]; however, it is now known that senescence can be induced by multiple other stimuli, including epigenetic changes, severe DNA damage, and the overexpression of certain oncogenes [228]. These senescence triggers can be either endogenous or exogenous in nature; for example, DNA lesions may be introduced by reactive oxygen species created during oxidative phosphorylation, or by therapeutic interventions such as radiation or chemotherapy [226]. One, several, or all of these phenomena may act in concert over time to generate a level of stress sufficient to trigger the cessation of cellular replication, first in individual cells and eventually in the entire cell population.

While senescence-causing stimuli may originate from diverse sources, they converge upon two major pathways: one governed by p16, the other by p53 (Figure 18). These proteins, both transcriptional regulators, are activated and stabilized in response to cellular stresses. p16 inhibits the CyclinD/CDK4,6 complex, preventing the phosphorylation of Rb. Unphosphorylated (activated) Rb is then free to interact with and suppress E2F, a transcription factor required for the expression of several genes necessary for cell cycle progression. p53, on the other hand, induces senescence by upregulating p21, a cyclin-dependent kinase (CDK) inhibitor that mediates cell cycle arrest by inhibiting the CyclinE/CDK2 complex. Phospho-Rb may also be downregulated by p21-mediated CDK inhibition; this overlap of the two senescence pathways increases the likelihood that stressful stimuli will result in cell cycle arrest [229]. The relative importance of the p53/p21 and p16/pRb pathways in establishing senescence varies by cell line; for example, BJ human foreskin fibroblasts senesce mainly through p53/p21, whereas p16/pRb predominates in IMR-90 human lung fibroblasts [180].

There is strong evidence that cellular senescence has evolved as a tumor suppressor mechanism, inducing potentially oncogenic cells such as those suffering from DNA damage, chromatin disruption, shortened or otherwise damaged telomeres, and other stresses to withdraw from the cell cycle rather than proliferating [230, 231]. In support of this view, immuno-
Figure 18. The major pathways governing mammalian cellular senescence. Cellular senescence is primarily controlled by the p16/Rb and p53 pathways, which are activated in response to various cellular stressors. p16 inhibits the CyclinD/CDK4,6 complex, preventing phosphorylation of Rb; Rb then binds to and inactivates the transcription factor E2F, resulting in cell cycle inhibition. p53 mediates cell cycle inhibition by upregulating p21, a CDK inhibitor affecting the CyclinE/CDK2 complex. Secondary p21-mediated inhibition of CyclinD/CDK4,6 provides crosstalk between the two pathways.
histochemical studies have demonstrated that human melanocytic nevi (birthmarks or moles) are comprised largely of senescent cells [232]. However, the anti-cancer protection conferred by senescence is a double-edged sword, as the presence of increasing numbers of senescent cells in an organism may contribute to the manifestation of age-related phenotypes and pathologies. It has been speculated that senescence is antagonistically pleiotropic — that is, it confers characteristics that are both beneficial and detrimental to organismal survival, depending on the circumstances — and may actually promote aging late in life [225, 228, 233]. Because they are resistant to apoptosis and unable to divide, senescent cells can contribute to the overall functional decline of an organism by impeding the replacement of damaged cells with new, healthy ones. Furthermore, senescent cells have been shown to overproduce various inflammatory cytokines and chemokines, proteases, and reactive oxygen species [226]. These materials are secreted to the extracellular space, inducing stress and contributing further to aging in the surrounding tissue.

Aging and senescence therefore appear to be integrally linked; in fact, the induction of senescence in cell culture through serial passage is one of the most widely-used models for mammalian aging. The model is of course imperfect; cultured cells are repeatedly exposed to non-physiologic levels of stresses such as trypsin, oxygen, and fluorescent light, and the two-dimensional nature of conventional culture alters intracellular signaling as well as extracellular matrix composition [234, 235]. Despite these drawbacks there is ample evidence that serially cultured cells do mimic many changes associated with in vivo aging, including genetic instability, decreased functional capacity and replicative capacity, and increased reactive oxygen species and oxidative damage [236–238]. The argument that senescence is a true physiological event has in particular been bolstered by recent findings that tissues from aging primates contain significant concentrations of cells displaying senescence-associated characteristics such as telomere dysfunction, upregulation of cell cycle inhibitors, and heterochromatization of the nuclear genome [239, 240]. Furthermore, the use of human cell lines which necessarily “express human genetic, metabolic, and regulatory behavior” [179] eliminates the need to pick out
mechanisms relevant to human aging from amongst the species-specific results often generated by animal-based studies. We chose to use a cellular senescence model of aging for this work because it provides an excellent way to conduct aging research — including the investigation of endogenous aging mechanisms as well as the effects of exogenous treatments added to the cell culture media — while avoiding the ethical and logistical difficulties which render robust, long-term aging studies on living humans all but impossible.

**Markers of Cellular Senescence**

As discussed above, senescent cells display characteristic features including growth arrest, resistance to apoptosis, and altered gene expression in addition to certain morphological changes. Growth arrest is often determined simply, if somewhat tediously, by cell counting: when the number of cells in a particular culture remains the same or decreases after an extended amount of time — commonly, around two weeks — the population is said to be senescent. The absence of DNA replication can also be confirmed by monitoring the incorporation of labeled nucleotides such as 5-bromodeoxyuridine and $^3$H-thymidine, or by immunostaining for DNA replication-associated proteins such as proliferating cell nuclear antigen (PCNA) and Ki-67. These assays, however, are incapable of confidently distinguishing senescence from the reversible form of growth arrest known as quiescence [241]. The study of aging has thus been stymied to some extent by a dearth of accurate senescence markers. In recent years several potential such markers have been identified and become commonly used in aging research. For this work, two of these — senescence-associated heterochromatin foci (SAHF) and senescence-associated $\beta$-galactosidase (SA-$\beta$-gal) — were chosen to serve as senescence indicators supplemental to the determination of growth arrest by cell counting. Details of their utility are discussed below.

**Senescence-Associated Heterochromatin Foci.** The condensed, inactive genetic material known as heterochromatin has roles in chromosome segregation, nuclear organization, and
gene silencing. Explicit evidence for its involvement in the control of senescence was first proposed in 2003 by Narita et al., who found that the DNA of human diploid fibroblasts induced to senesce by a variety of means had a characteristic punctate appearance as visualized by DAPI staining (Figure 19). These foci, which were not found in proliferating or quiescent fibroblasts, contained no active sites of transcription and featured hypoacetylated histones, histone H3 methylated on lysine 9, and the heterochromatin protein HP1. They were thus found to be similar in nature to heterochromatin, and were dubbed “senescence-associated heterochromatin foci,” or SAHF. Senescent cells were found to contain SAHF at the promoters of several E2F target genes necessary for cell proliferation; it was therefore theorized that the formation of heterochromatin at these sites promotes cell cycle arrest by preventing E2F binding. In support of this idea, it was confirmed that E2F-responsive genes are stably repressed in senescent cells [180]. It is likely that other genes are similarly repressed; DNA from senescent cells is much less susceptible to digestion by micrococcal nuclease than is DNA from quiescent or proliferating cells [242]. As Narita et al. found SAHF formation to be dependent upon Rb, these foci are significantly more pronounced in cell lines with p16/Rb-mediated senescence (e.g. IMR-90 human lung fibroblasts) than in cell lines whose senescence is primarily controlled by p53/p21 (e.g. BJ human foreskin fibroblasts, mouse embryonic fibroblasts) [243, 244]. Nevertheless, it was recognized that SAHF showed great promise as indicators of senescence, as they appear to

be highly specific to senescent cells, they are easily detected, and there is already some understanding of their mechanism of formation and role in cell cycle arrest [180, 181]. As they have quickly become one of the most reliable senescence biomarkers, they were an obvious choice for use in this work.

While SAHF are somewhat similar in appearance to apoptotic bodies (the small, membrane-enclosed vesicles formed by cells undergoing apoptosis), there are several differences which may be used to distinguish between these structures. The duration of their existence is perhaps the most obvious; whereas apoptotic bodies form quickly during the degradation stage of apoptosis and are evident for only several hours before being phagocytosed by neighboring cells [245], SAHF form over 7-12 days [181] and may persist for weeks. Furthermore, as apoptosis is a rare event in cell populations that have not been subjected to cytotoxic stimuli, only a small number of apoptotic cells will generally be seen at any given time [245]. SAHF, on the other hand, have been found in around 80% of cells from a typical senescent population [180]. Finally, SAHF do not exhibit the post-translational modification patterns typical of apoptotic DNA, such as phospho-H3(Ser10), phospho-H3(Ser28), phospho-H2B(Ser14), phospho-H3(Ser10), and phospho-H3(Ser28) [246]. In this work, DAPI foci were identified as SAHF based upon their duration and frequency.

**Senescence-Associated β-Galactosidase.** β-galactosidase is a lysosomal enzyme normally involved in the cleavage of galactose residues from macromolecules such as glycoproteins and sphingolipids [247]. In 1995, a study by Dimri et al. suggested that senescent cells express a modified form of beta-galactosidase with optimal activity at pH 6.0 (Figure 20). This activity was found in senescent but not presenescent, quiescent, or immortalized human fibroblasts, and was also found to correlate with replicative age in skin samples from adult donors. The authors concluded that activity of this putative enzyme variant, which became known as senescence-associated beta-galactosidase, is a specific biomarker for senescence [248]. As the assay is rapid, inexpensive, and simple to perform, it was soon being extensively used as an in-

dicator of senescence in cell culture studies. However, neither the origin of SA-β-gal activity nor its role in senescence was understood.

Controversy flared when subsequent studies questioned the assay’s specificity and applicability to different cell types. For example, Going et al. found significant SA-β-gal activity in both proliferating and non-proliferating cells from human gastrointestinal tissue, and in contrast to the results of Dimri et al., they found no correlation between beta-galactosidase activity at pH 6.0 and donor age [249]. Moreover, they consistently detected higher activities at pH 4.0 than at pH 6.0 in all cell types tested, suggesting the possibility that SA-β-gal is not in actuality a specific enzyme variant but rather represents the residual activity of ordinary lysosomal β-galactosidase at a suboptimal pH. These findings were echoed by several other groups [250, 251], perhaps most notably by Lee et al. who found that senescent fibroblasts with inhibited or defective lysosomal β-galactosidase did not express SA-β-gal activity [252]. It was also discovered that SA-β-gal expression could be triggered by conditions other than senescence, including H₂O₂ treatment, serum starvation, and extended periods of confluence [250, 251, 253].

Where does this leave SA-β-gal’s status as a biomarker of senescence? It seems that even the group initially proposing its utility now concedes that SA-β-gal is lysosomal in origin [241]. However, increased lysosomal activity has long been associated with cellular senescence and aging [250–252]. For this reason, SA-β-gal expression does correlate with cellular age in many cell types under certain circumstances. It is thus reasonable to consider SA-β-gal a surrogate
marker for senescence, with the following caveats:

1. SA-β-gal expression alone may not be used to define senescence. Confirmation by other means (e.g. determination of growth arrest) is required.

2. SA-β-gal should not be used to indicate senescence if cells have been serum-starved, treated with \( \text{H}_2\text{O}_2 \), or grown with prolonged confluence. In order to maximize SA-β-gal’s utility as a marker, cells should be consistently subcultured at 70-80% density with regular media changes.

3. As SA-β-gal expression can vary by cell line, it should not be used to compare relative cellular age between different cell types.

These guidelines were followed strictly in this work in order to minimize the possibility of producing misleading data.

**Nicotinamide-Mediated Lifespan Extension**

Nicotinamide, a component of vitamin B\(_3\), is an NAD precursor that has been shown to increase cell proliferation and enhance cell survival in the face of challenges such as physical damage and oxidative stress [254–256]. Matuoka *et al.* reported in 2001 that treating human fibroblasts with nicotinamide induces a reversal of several senescence-related phenotypes, including morphological changes and SA-β-gal expression, but does not affect replicative lifespan [257]. These experiments, however, were performed using near-senescent cells. Subsequent work in our laboratory [191] demonstrated that replicative lifespan is extended significantly if prolonged NAM supplementation is begun when cells are relatively young (around 32 population doublings or PDs, as opposed to 64 in the Matuoka study). This result was corroborated by a concurrent report from Kang *et al.*, who found that nicotinamide not only increased replicative lifespan but attenuated SA-β-gal expression, decreased levels of senescence-associated proteins such as p53 and p21, reduced the rate of telomere shortening, and relieved the aging-associated accumulation of reactive oxygen species (ROS) [258].
The mechanism of NAM-mediated lifespan extension is not currently understood. Other chemicals that have been shown to delay senescence, such as kinetin, carnosine, and N-\(t\)-butyl hydroxylamine, display antioxidant or ROS-scavenging activities [259–261]. Cells rapidly enter senescence upon withdrawal of these treatments. NAM, on the other hand, is not itself an antioxidant, and its effects have been shown to persist for up to 20 PDs after its removal from the media [258]. It therefore appears that NAM is unique among lifespan-extending chemical agents in that its activity, at least in part, stems from something other than acute modulation of cellular conditions. We hypothesize a role for PARP-1 in this process. As NAM is one of the reaction products of poly(ADP-ribosyl)ation, it inhibits PARP-1 through negative feedback regulation[192]. Theoretically, PARP inhibition could lead to lifespan extension by a number of mechanisms, including inhibition of PARP-mediated p53 and NF-\(\kappa\)B activation, relief of energy depletion caused by PARP activation in aging cells experiencing amounts of DNA damage, inhibition of AIF-mediated apoptosis, or even modulation of DNA methylation. However, the literature strongly suggests a positive correlation between PARP activity and organismal longevity [171, 172]. We submit that analysis of PARP-1 and poly(ADP-ribosyl)ation levels in NAM-treated aging cells could resolve this apparent paradox while simultaneously shedding some light upon both the mechanism of NAM-mediated lifespan extension and the role of PARP-1 in the establishment of senescence.

**Experimental Procedures**

**Materials and reagents.** Unless stated otherwise, reagents were purchased from either Sigma-Aldrich or Fisher Scientific. Cell culture reagents were purchased from Invitrogen, ATCC, or Atlanta Biologicals. Flasks and plates for cell culture were supplied by Corning. All cell lines were obtained from ATCC.

**Mammalian cell culture.** All cell manipulations were performed in a laminar flow hood under aseptic conditions. IMR-90 human lung fibroblasts and BJ human foreskin fibroblasts were
grown in 75 or 150 cm² tissue culture flasks and maintained in a 37°C incubator at 5% (v/v) CO₂ in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. NAM (5 mM) prepared from a 1 M filter-sterilized stock solution was added as indicated. Medium was replaced every 3-4 days. Cells were subcultured at 70-85% confluency by decanting the media, rinsing the flask with PBS, and adding 0.25% (w/v) trypsin-EDTA (2-3 mL, prewarmed to 37°C). The cells were incubated at 37°C for 5-10 minutes until cell detachment occurred. Trypsin was neutralized by addition of supplemented DMEM to a final volume of 10 mL and cells were seeded in fresh flasks at a 1:3 to 1:8 ratio. During subculturing, a portion of cell suspension was combined with an equal volume of trypan blue live/dead stain, incubated for 1-2 minutes, and then counted using a hemocytometer to determine the total number of live and dead cells. Percent cell viability was calculated using the following formula, where \( S \) = number of stained cells and \( U \) = number of unstained cells:

\[
V = \left( \frac{S}{S+U} \right) (100\%)
\]

Population doubling was calculated using the following formula, where \( H \) = the number of cells harvested, \( S \) = the number of cells seeded, and \( X \) = the number of PDs elapsed since the previous count:

\[
\log H - \log S = X (\log 2)
\]

Approximately every 5 PDs, several 2 mL aliquots of cells were prepared in supplemented DMEM containing 8% (v/v) DMSO as a cryoprotectant. Cells were frozen overnight at -80°C, then stored under vapor phase nitrogen. To start cultures from frozen stock, cells were thawed quickly in a 37°C waterbath and transferred to a T-75 flask containing approximately 20 mL supplemented DMEM. Medium was replaced after 24 hours to remove cryoprotectant.

**Cell proliferation assay.** BJ cells were seeded 50,000 per well with 2 mL supplemented DMEM in 6-well plates. NAM was added to the media at 0, 5, 10, or 20 mM. Cells were allowed to
proliferate for 6 days, then trypsinized and counted as previously described.

**Mammalian cell harvesting.** IMR-90 or BJ cells growing in culture were trypsinized as previously described, transferred to a 15 mL centrifuge tube, and centrifuged at 300 x g for 5-10 min in order to pellet the cells and decant off the medium. The cell pellet was subsequently washed with 10 mL PBS, centrifuged and decanted as described above before storing the tube on its side at -80° C until further analysis.

**Cell extract preparation.** Whole cell lysates were prepared by resuspending IMR-90 or BJ cell pellets in 1 mL of either NET-N buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% v/v NP-40) or EBC buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 2 mM EDTA, 0.5% v/v NP-40) and incubating on ice with occasional agitation for 30 min. Proteases were inhibited during lysis using Roche's Complete Mini Protease Inhibitor Cocktail Tablets according to manufacturer's instructions. Protein concentrations were determined by the Bradford method [209] with a BSA standard using Pierce’s Coomassie Plus reagent kit.

**Cytochemical detection of PARP activity.** IMR-90 or BJ cells growing in culture were trypsinized as described previously and seeded 500,000 per well with 2 mL supplemented DMEM in 6-well plates. Cytochemical detection of PARP activity was performed 24 hrs after seeding, essentially as described by [212]. Supplemented medium was removed and replaced with 2 mL per well of either unsupplemented DMEM as a control or unsupplemented DMEM containing 0.5 mM H$_2$O$_2$ to stimulate PARP activity. Cells were incubated for 30 min at 37° C after which medium was removed and replaced with 2 mL per well PARP reaction buffer containing 56 mM HEPES pH 8.0, 28 mM KCl, 28 mM NaCl, 2 mM MgCl$_2$, 0.01% (w/v) digitonin for cell permeabilization, and 12.5 µM biotinylated NAD (Trevigen). After a 30 min incubation at 37° C, reaction buffer was removed and cells were fixed at -20° C with ice-cold 95% (v/v) ethanol (1 mL/well, 10 min) followed by ice-cold TCA (100% v/v, 1 mL/well, 10 min). Cells were rinsed in PBS and endogenous peroxidase activity was blocked with 0.5% (v/v) H$_2$O$_2$ in methanol (1
mL/well, 15 min). After two 5-min PBS washes, cells were blocked in 1% (w/v) BSA/PBS for 30 min followed by two 5-min rinses in 0.1% (v/v) Triton X-100/PBS. Incorporated biotin was detected with a 1:100 (v/v) dilution of streptavidin-HRP (Upstate) in 0.1% (v/v) Triton X-100/PBS for 1 hour at 37°C. Cells were washed four times with 0.1% (v/v) Triton X-100/PBS (2 mL/well) and color was developed using SigmaFast Enhanced DAB substrate tablets (Sigma) according to the manufacturer’s instructions. To stop the reaction, substrate was removed and cells were washed briefly with 2 mL PBS per well. Cells were overlaid with glycerol and phase contrast images were taken on a Nikon TMD Diaphot microscope using the 40x Ph1 objective.

**Quantitation of PARP activity by cellular ELISA.** IMR-90 and BJ fibroblasts were seeded 10,000 per well in a 96-well plate (Corning). Medium was removed 24 hrs after seeding and replaced with either unsupplemented DMEM as a control or unsupplemented DMEM containing 5 mM H₂O₂ to stimulate PARP activity. The plate was incubated for 30 min at 37°C after which the cellular ELISA was performed essentially as described by Bakondi et al. [212]. Briefly, medium was replaced with 100 µL/well of a PARP reaction buffer containing 56 mM HEPES pH 8.0, 28 mM KCl, 28 mM NaCl, 2 mM MgCl₂, 0.01% (w/v) digitonin for cell membrane permeabilization, and 12.5 µM biotinylated NAD (Trevigen) and incubated for 30 min at 37°C. PARP reaction buffer was replaced with 200 µL unsupplemented DMEM. Ice-cold 50% (v/v) TCA was added to a final concentration of 10% and cells were fixed at room temperature for 30 min. Endogenous peroxidase activity was blocked with 0.5% (v/v) H₂O₂ in methanol (100 µL/well, 15 min) and nonspecific binding sites were blocked with Starting Block (Pierce) according to manufacturer’s instructions. PAR-incorporated biotin was detected by incubation with a 1:500 (v/v) dilution of streptavidin-HRP (Upstate) in Starting Block containing 0.05% (v/v) Tween-20 for 1 hour at 37°C followed by extensive washing with PBS and addition of 50 µL per well TACS-Sapphire (Trevigen). The plate was incubated in the dark for 1 hour after which the chromogenic reaction was stopped by addition of 0.2 N HCl (50 µL/well). Absorbance at 450 nm was read on a Tecan microplate reader.
DAPI staining for senescence-associated heterochromatin foci. IMR-90 and BJ fibroblasts were cultured on sterile coverslips resting in 6-well plates to 70-80% confluence. Media was removed and coverslips were washed twice briefly with 1 mL/well PBS. Cells were fixed in 2% (w/v) formaldehyde/PBS solution for 5 min at room temperature, then washed twice for 5 min with 1 mL/well PBS. Approximately 300 µL of 300 nM DAPI (Invitrogen) in PBS was added to each coverslip and cells were incubated for 5 min at room temperature. The staining solution was aspirated and coverslips were washed twice briefly with 1 mL/well PBS. Coverslips were rinsed in deionized water, allowed to dry, and affixed to glass microscope slides using Prolong Gold (Invitrogen) according to the manufacturer’s instructions. Images were obtained on a Deltavision deconvolution microscope equipped with a DAPI filter and a 100x oil immersion objective.

Senescence-associated β-galactosidase activity staining. SA-β-gal staining was performed essentially as described by Dimri et al. [248]. IMR-90 and BJ fibroblasts were cultured on sterile coverslips resting in 6-well plates to 70-80% confluence. Media was removed and coverslips were washed three times briefly with 2 mL/well PBS. Cells were fixed in 1 mL/well fixation solution (0.2% w/v glutaraldehyde and 2% v/v formaldehyde in PBS) for 5 min at room temperature, then washed twice for 5 min with 2 mL/well PBS. Cells were stained with 1 mL/well staining solution (40 mM citric acid/sodium phosphate buffer pH 6.0, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 150 mM NaCl, 1 mg/mL X-gal) overnight at 37° C. The staining solution was aspirated and cells were washed in 2 mL/well PBS. Coverslips were rinsed briefly in deionized water and glycerol mounted on glass coverslips. Phase contrast images were taken on a Nikon TMD Diaphot microscope using the 40x Ph1 objective.

Results and Discussion

Nicotinamide increases cell proliferation in BJ skin fibroblasts. Previous work in our lab had demonstrated that NAM treatment increases both cell proliferation and replicative lifespan in
IMR-90 fibroblasts[191]; however, the effect of NAM upon BJ fibroblasts was unknown. In order to confirm that NAM would have a similar effect on BJ cells and determine the optimum concentration for treatment, we performed a cell proliferation assay (Figure 21). Equal numbers of young BJ cells (PD 31) were seeded and grown in the presence of various concentrations of NAM (0, 5, 10, or 20 mM) for one week, after which the cells were trypsinized and counted. 5 mM NAM had the most pronounced effect, inducing a roughly 3-fold increase in cell number compared to the control, whereas 10 mM induced a 2-fold increase and 20 mM showed no effect. As these results were similar to those achieved with NAM treatment of IMR-90 cells, it was considered likely that 5 mM NAM would increase the replicative lifespan of BJ cells as well. We therefore chose to use this NAM concentration in subsequent experiments to perturb the induction of senescence in IMR-90 and BJ fibroblasts.

**Nicotinamide extends the replicative lifespan of primary human fibroblasts.** In order to obtain samples for the analysis of PARP-1 protein levels and enzymatic activity during cellular aging and NAM-mediated replicative lifespan extension, IMR-90 and BJ cells were cultured in the presence or absence of 5 mM NAM from a relatively early passage number up through the nor-

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**Figure 21. Nicotinamide increases cell proliferation in BJ fibroblasts.** Young BJ skin fibroblasts were allowed to proliferate in the presence or absence of NAM (5, 10, or 20 mM). After 1 week cells were trypsinized, resuspended in 10 mL supplemented DMEM, and counted. Both 5 mM and 10 mM NAM induced a marked increase in cell proliferation. *p < 0.05, error bars represent standard deviation.
mal senescence point for each cell line. Preventing the induction of premature aging due to suboptimal cell culture conditions was crucial; media was therefore consistently replenished every 3-4 days to maintain nutrient levels and remove metabolic byproducts, and cells were split at 70-80% confluency to avoid overgrowth. Cell counts were performed at each passage and an ongoing plot of PDs vs. days of culture was maintained for each line. In addition, age-matched samples of live cells as well as frozen pellets for the preparation of whole cell lysates were generated at three different time points — henceforth referred to as “young,” “middle-age,” and “old” — during this process (Table 7).

Cell growth plots revealed that NAM markedly increased the replicative lifespan of both fibroblast types tested. Replicative lifespan increased from 54 PDs to more than 60 PDs in IMR-90 cells (Figure 22A, an 11% increase) and from 59 to more than 68 PDs in BJ cells (Figure 22B, a 15% increase). Our laboratory has now demonstrated NAM-mediated upregulation of cell proliferation and replicative lifespan extension in three lines of primary human fibroblasts, including cells with p16/Rb-dependent senescence (WI-38 and IMR-90 lung fibroblasts) and cells with p53/p21-dependent senescence (BJ foreskin fibroblasts). Taken together with the results of Kang et al., who demonstrated a similar response in another distinct line of primary human skin fibroblasts [258], this work suggests that NAM-mediated lifespan extension may be a phenomenon common to many different cell types.

Table 7. Actual age of cells used for young, middle-age, and old time points. For each cell line, collected samples were age-matched to within 2-3 PDs. “Young” cell samples were collected as soon as possible after the establishment of the culture. “Old” cell samples were collected at the experimentally-determined senescence point for control cells (around 54 for IMR-90 and 59 for BJ). “Middle-age” cell samples were collected at the approximate midpoint between young and old age.

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Nicotinamide extends the replicative lifespan of primary human fibroblasts. Young primary human fibroblasts were allowed to proliferate in the presence or absence of 5 mM NAM. Cells were subcultured and counted at 70-80% confluency and population doublings vs. time was plotted. At each cell line's normal senescence point, NAM-treated cells were found to have surpassed the usual replicative lifespan; IMR-90 by 6 population doublings, or 11% (A), BJ by 9 population doublings, or 15% (B).

Nicotinamide delays the appearance of senescence markers in primary human fibroblasts.

The replicative lifespan extension observed in NAM-treated primary human fibroblasts was accompanied by a delay in the appearance of senescence markers. DAPI staining of old IMR-90 and BJ cells revealed the presence of SAHF in the nuclei (Figure 23B and E) as compared to the relatively diffuse chromatin staining seen in young cells from both lines (Figure 23A and D). The appearance of SAHF was greatly reduced, however, in NAM-treated old IMR-90 cells (Figure 23C) and almost completely abrogated in NAM-treated old BJ cells (Figure 23F). (The more subtle overall appearance of SAHF in BJ fibroblasts results from the predominance of the p53/p21 senescence pathway in that cell line; as SAHF formation is largely p16/Rb-dependent, heterochromatin foci development is less prominent in cells whose senescence is governed primarily by p53/p21 [180].)

Similarly, NAM treatment attenuated the expression of senescence-associated β-galactosidase. Cells positive for SA-β-gal activity were very infrequent in young IMR-90 and BJ populations (Figure 24A and D), but ubiquitous in old ones (Figure 24B and E). Staining was greatly reduced in age-matched NAM-treated cells, though the two tested cell lines behaved somewhat differently. Old NAM-treated IMR-90 cells featured markedly more diffuse but still relatively widespread staining (Figure 24C), whereas SA-β-gal activity was almost com-
Figure 23. Nicotinamide delays senescence-associated heterochromatin foci formation in primary human fibroblasts. Representative images of young and old IMR-90 and BJ fibroblasts after fixation and DAPI staining. The chromatin of old cells displays the characteristic punctate appearance of SAHF (B and E) as compared to the more uniform-looking chromatin of young cells (A and D). SAHF formation is greatly reduced in age-matched old cells grown in NAM-supplemented medium (C and F), indicating a delay in the establishment of senescence.
pletely abrogated in old NAM-treated BJ cells (Figure 24F). The cause of this difference is unknown. It is possible that the IMR-90 cell line simply has higher overall lysosome activity or a more pronounced age-related increase in lysosomal number than the BJ cell line. As it now appears that “senescence-associated” β-galactosidase activity originates from ordinary lysosomal β-galactosidase [250, 252], this could account for the more frequent appearance of SA-β-gal staining in old NAM-treated IMR-90 cells. A comparative study of young, old, and old NAM-treated BJ and IMR-90 cells using a lysosome-specific stain such as Lysotracker Red to visualize changes in cellular lysosomal mass over time [262] would provide evidence to support or refute this hypothesis.

Alternatively, it is possible that NAM supplementation has a stronger effect in BJ than in IMR-90 cells, causing a more pronounced delay in the appearance of senescence markers (as well as a larger increase in replicative lifespan, as seen in Figure 22). Since senescence in BJ fibroblasts is primarily governed by p53/p21 rather than p16/Rb, this could be an indication that NAM-mediated lifespan extension acts at least in part through the p53 pathway. Next steps in the evaluation of this hypothesis would include analysis of p53 and p16 protein levels in aging cells ±NAM, as well as additional studies to determine whether the magnitude of NAM-induced lifespan extension is consistently higher in fibroblast lines with p53/p21-dependent senescence.

**Nicotinamide markedly increases oxidative stress-induced PARP activity in primary human fibroblasts, but not baseline PARP activity.** To begin exploring a possible role for PARP-1 in nicotinamide-mediated lifespan extension, we analyzed the poly(ADP-ribosyl)ation response in aging IMR-90 and BJ fibroblasts (±NAM) using a PARP activity assay. Cells were cultured in 96-well plates and incubated with a PARP reaction buffer containing biotinylated NAD (bio-NAD). The biotin label was incorporated into newly-synthesized PAR, which could then be quantitated colorimetrically using a streptavidin-HRP detection system. A major strength of this assay lies in its ability to quantitate “housekeeping” levels of PARP activity as well as ac-
Figure 24. Nicotinamide reduces expression of senescence-associated β-galactosidase in primary human fibroblasts. A-F, representative images of young and old IMR-90 and BJ fibroblasts after fixation and staining for SA-β-gal activity [248]. While SA-β-gal staining is very infrequent in young cells (A and D), most of the senescent cells are positive for SA-β-gal activity (B and E). Staining is more widespread but much more diffuse in age-matched old NAM-treated IMR-90 cells (C); it is almost completely abrogated in age-matched old NAM-treated BJ cells (F). Percentages of IMR-90 and BJ cells demonstrating positive SA-β-gal staining are given in panels G and H, respectively. Cells from three different images were counted for each time point. ***p < 0.00001, **p < 0.0001, *p < 0.05.
tivity that occurs in response to a particular stimulus. In this case for example, we induced oxidative damage with 0.5 mM H$_2$O$_2$ in one set of cells prior to bio-NAD incubation. This allowed us to compare baseline levels of PARP activity to those observed during a DNA damage response. Cytochemical PARP activity staining was also performed using a similar method, but with DAB detection in place of colorimetric quantitation (Figure 26).

As shown in Figure 25, neither age nor NAM treatment significantly affected baseline PARP activity in IMR-90 (A) or BJ cells (C). Considering that NAM is a known PARP-1 inhibitor,
this result was somewhat surprising. A large body of work has demonstrated the protective effects of NAM against cell death in a number of disease states often attributed to PARP overactivation, including neuronal excitotoxicity and ischemia/reperfusion injury [263]. Furthermore, NAM has been shown to block the production of inflammatory mediators [192]. As resistance to cell death and downregulation of inflammation are both characteristics of PARP-1 deficient cells [264], NAM’s beneficial effects have commonly been assumed to result from its action as a PARP inhibitor. Our findings indicate that this may not be the case. The results of PARP activity analysis in H$_2$O$_2$-treated cells were even more striking. Not only was there no evidence of PARP inhibition, but to the contrary, oxidative-damage-mediated PARP activity was markedly increased in NAM-treated IMR-90 and BJ cells (Figure 25C and D). Despite NAM’s status as a known PARP inhibitor, it does not appear to be acting as one in this case. NAM-mediated lifespan extension must therefore occur through some other mechanism.

Figure 25 also illustrates an increase in oxidative-stress-mediated PARP activity with age in both tested cell lines. This result is consistent with previous studies demonstrating an age-related decline in the function of antioxidant enzymes such as superoxide dismutase and catalase [265]. Aged cells also have higher overall levels of ROS than young cells due to “leaking” of electrons from damaged mitochondria during oxidative phosphorylation [266]. They are therefore less likely than younger cells to successfully neutralize ROS generated from exogenous sources, as their faltering defense systems are already overwhelmed by high levels of endogenous ROS. This leads to increased oxidative DNA damage, and thus increased PARP activity in the H$_2$O$_2$-treated cells. It should be noted, however, that no significant age-related increase in housekeeping PARP activity was observed in this experiment despite the fact that the overall amount of oxidative expressed experienced by a cell tends to increase with age. While this result at first appears puzzling, it is likely explained by the fact PAR chains generated under moderate levels of oxidative stress — as opposed to the high levels induced by acute H$_2$O$_2$ treatment — tend to be relatively short [17]. Increases in cellular PAR due to the formation of these short chains may therefore fall below the detection limit of the bio-NAD cellular ELISA
Figure 26. Cytochemical PARP activity staining. Young and old IMR-90 and BJ fibroblasts (+/- NAM) were cultured on coverslips, treated with 0.5 mM H$_2$O$_2$ to stimulate PARP activity, and incubated with a PARP reaction buffer containing biotinylated NAD. Newly-synthesized PAR was then visualized by streptavidin-HRP detection followed by DAB staining. Representative images from IMR-90 cells (A-G) and BJ cells (G-L) are shown.
method. Analysis of the poly(ADP-ribosyl)ation response using a radiolabeled NAD method instead could provide the sensitivity necessary to observe significant changes in baseline PARP activity with age and/or NAM treatment.

**Increased oxidative-damage-mediated PARP activity in NAM-treated cells indicates a role for the NAD salvage pathway.** We had initially hypothesized that PARP inhibition may be the mechanism behind NAM-mediated lifespan extension; however, our experimental results clearly indicate otherwise. Why would oxidative-damage-mediated PARP activity increase when cells are treated with a PARP inhibitor? The answer to this apparent conundrum may lie in the NAD salvage pathway. While mammalian cells can synthesize NAD *de novo* from tryptophan, the salvage of nicotinamide-ring-containing precursors originating from dietary sources or poly(ADP-ribosyl)ation reaction byproducts is critical to cellular survival and organismal health [267]. In NAD salvage, NAD is synthesized from these molecules through adenylation and phosphoribosylation reactions: NAM is first converted to nicotinamide mononucleotide (NMN) by the rate-limiting enzyme nicotinamide phosphoribosyl transferase (NAMPT), and the resulting NMN is then converted to NAD by nicotinamide mononucleotide adenyllyl transferases (NMATs) (Figure 27). It is therefore conceivable that NAM supplementation would lead to increased cellular NAD and ultimately to increased poly(ADP-ribosyl)ation, as PARP activity is highly dependent upon NAD concentration.

![Figure 27. The mammalian NAD salvage pathway.](image)

NAM is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyl transferase (NAMPT), the rate-limiting enzyme of the pathway. NMN is then coverted to NAD by nicotinamide mononucleotide adenyllyl transferases (NMATs) [268].
An examination of the literature hints that this is indeed the case. *In vitro* studies have shown that the IC\textsubscript{50} of NAM in isolated PARP-1 is around 30 µM. However, the concentrations at which NAM exerts *in vivo* effects in whole cells are much higher — on the order of 5-30 mM — and NAM dosages in the range of hundreds of mg/kg have been required to produce protective effects in animal models of disease states such as stroke and shock [263]. Furthermore, studies of 3-aminobenzamide, a PARP inhibitor with an *in vitro* potency similar to that of NAM, have demonstrated only partial inhibition of poly(ADP-ribosyl)ation *in vivo* [269]. Taken together, these observations suggest both that NAM’s *in vivo* effects originate from a mechanism other than PARP inhibition, and that this mechanism can be triggered by supplementing cells or organisms with NAM concentrations far above those necessary to inhibit PARP. This scenario is consistent with the hypothesis that NAD salvage is involved: if enough NAM is supplied, the effects of the resulting increase in cellular NAD may supersede and mask those of NAM-mediated PARP inhibition. Indeed, there are multiple reports of increased cellular NAD resulting from NAM supplementation; however, such results have often been errantly associated with reduced consumption of NAD by inhibited PARP-1 [256, 270, 271].

**Abundant cellular NAD resulting from increased flux through the salvage pathway is a plausible mechanism for NAM-mediated replicative lifespan extension.** Acceptance of a possible link between the NAD salvage pathway and mammalian longevity is increasing. Recent reports demonstrate that overexpression of the rate-limiting enzyme NAMPT extends the lifespan of human cells [272, 273], and it has even been speculated that NAMPT is a functional equivalent of PNC1, a “master longevity regulatory gene” in yeast [274]. Below, we suggest two mechanisms whereby increased cellular NAD resulting from processing of supplemented NAM by the NAD salvage pathway could induce replicative lifespan extension.

**Prevention of PARP-mediated NAD depletion.** The “PARP suicide hypothesis,” in which massive DNA damage causes PARP-1 overactivation and subsequent NAD depletion, energy failure, and cell death, was first advanced over 20 years ago [103]. While it has since become
clear that there is much more to the story, PARP-1 is still acknowledged as a voracious consumer of NAD with the potential to substantially decrease cellular NAD levels. Meanwhile, aging cells face increasing amounts of DNA damage which must be repaired if apoptosis and/or senescence are to be avoided. This causes a dilemma: stimulation of the DNA repair response by increasing amounts of damage leads to increased PARP activity, but that activity — while necessary for efficient DNA repair — reduces the availability of NAD for other important cellular tasks. Increasing the cellular NAD pool by NAM supplementation could therefore contribute to cellular lifespan extension by permitting the PARP-mediated repair of large amounts of DNA damage while ensuring that the cellular NAD concentration remains high. This hypothesis is supported by the fact that PARP-mediated DNA damage repair capacity is positively correlated with organismal longevity [171, 172].

**Upregulation of SIRT1.** Sir2 (known as SIRT1 in mammals) is an NAD-dependent protein deacetylase associated with lifespan extension in yeast, worms, and flies [275]. Its longevity-promoting effect in lower organisms is undisputed; however, its impact in mammals has been uncertain. While several reports have suggested minor SIRT1-mediated lifespan extension in mouse and human cells, many groups, including our own, have found no effect whatsoever [191, 276]. This has been a topic of some confusion and controversy in the aging research community.

A recent report [277] appears to explain these conflicting results. When Ho et al. overexpressed SIRT1 alone in human muscle cells, minimal change was observed. However, SIRT1 overexpression accompanied by simultaneous overexpression of NAMPT — which is known to decrease with age [272] — induced a dramatic increase in replicative lifespan. This striking result suggests that SIRT1’s ability to extend lifespan is blocked by the limited availability of NAD in aging cells, and that this block can be removed if the age-related decline in NAD salvage is overcome (e.g. by overexpressing the rate-limiting enzyme in the salvage pathway). NAM supplementation can be thought of as an alternative way of accomplishing the same end result;
the presence of abundant substrate for NAMPT may compensate for age-related decline in the efficiency of the salvage pathway, resulting in an increased cellular NAD pool and subsequent upregulation of SIRT1. While the mechanism by which SIRT1 extends lifespan is not completely understood, it has been found to deacetylate (and thus deactivate) p53, thereby antagonizing p53-dependent senescence and apoptosis [278, 279]. If NAM-mediated lifespan extension is in fact controlled in part by SIRT1’s regulation of p53, this could explain our observation of a more robust NAM-mediated lifespan extension in BJ cells, whose senescence is primarily controlled by p53, than in IMR-90 cells in which the p16/Rb pathway predominates.

**Conclusion**

The objective of this work was to explore a possible role for PARP-1 in the replicative lifespan extension of primary human fibroblasts by NAM. We initially hypothesized that NAM-mediated PARP inhibition was the mechanism behind this phenomenon; however, our results demonstrated increased rather than decreased poly(ADP-ribosyl)ation activity in NAM-treated cells, ruling out PARP inhibition and instead suggesting involvement of the NAD salvage pathway. We now propose that supplemented NAM is taken up by the NAD salvage pathway, ultimately leading to increased cellular NAD and extending replicative lifespan by both preventing PARP-mediated NAD depletion and upregulating SIRT1 (Figure 28).

Subsequent experiments that would support and strengthen this hypothesis are as follows:

1. **Confirm that NAM supplementation leads to increased cellular NAD.** Determine the cellular concentrations of NAD to confirm that exogenous NAM is being taken up by the NAD salvage pathway. This can be accomplished with an enzyme cycling assay in which NAD is repeatedly reduced and oxidized by way of coupled enzyme reactions, producing a measurable chromagen in the process [271].
**Figure 28.** Proposed mechanism for nicotinamide-mediated lifespan extension in human primary fibroblasts. Exogenous NAM is processed by the NAD salvage pathway, leading to increased levels of cellular NAD. Lifespan extension results from increased capacity for PARP-1-mediated DNA repair without danger of cellular NAD depletion, as well as upregulation of SIRT1 and subsequent downregulation of p53-mediated senescence and apoptosis.
2. Provide evidence that NAM treatment increases SIRT1 activity. Analyze SIRT1 protein levels via Western blot and measure SIRT1 deacetylase activity using a fluorometric assay to determine whether SIRT1 is upregulated by NAM treatment.

3. Provide evidence that NAM treatment modulates p53 activity. Analyze levels of acetylated p53 via Western blot to look for downstream effects of SIRT1 upregulation. Also analyze levels of p53/p21 and p16/Rb pathway proteins; if NAM-mediated lifespan extension is controlled in part by SIRT1 modulation of p53, one would expect to see downregulation of p53 and p21 in NAM-treated cells. Perform lifespan assays with additional cell types to determine whether cell lines with p53-dependent senescence consistently display a more robust NAM-mediated lifespan extension effect.

In addition to providing a possible mechanism for NAM-mediated lifespan extension in primary human fibroblasts, this work highlights the need for caution when designing and interpreting experiments performed using nicotinamide. Because NAM is a known PARP inhibitor, a number of reports have attributed its protective effects in various disease models to PARP inhibition. Surprisingly, many of these studies included no actual quantitation of cellular poly(ADP-ribose); it was merely assumed that PARP activity must have been decreased by NAM treatment since protection against NAD depletion was observed. When interpreting experimental results it is important to keep in mind that as a precursor of NAD, NAM is capable of exerting a multitude of effects beyond those of simple PARP inhibitors. Failure to do so can both result in misleading conclusions and obscure promising leads for future research.
Appendix: Evaluation of Cell Cultures for Mycoplasma Contamination

Introduction

Types of Cell Culture Contamination. While cell culture is undeniably a powerful tool for biological research, it can be quite challenging to work with in the laboratory. Cells growing in culture are no exception to the rule that living organisms must respond to environmental changes. A wide range of factors including UV exposure, fluctuations in temperature and pH, static electricity buildup on plasticware, and the vibrations that emanate from incubators and ventilation systems can cause erratic growth patterns, attachment problems, and even cell death [280]. Unlike other types of laboratory equipment failures, the complete loss of a culture cannot be solved with a call to the repair technician; it is often catastrophic to an experiment, especially in the case of aging studies where cells are grown continuously for months on end.

Biological contamination is one of the most common — and most feared — cell culture mishaps. Even researchers who consistently practice good aseptic technique will experience it from time to time, as sources of contamination are ubiquitous in the laboratory. Foreign organisms such as bacteria and fungi can easily be introduced by way of dirty waterbaths and incubators, improperly sterilized pipettes, malfunctioning laminar flow hoods, or the investigator’s clothing. Fortunately, both bacterial and fungal contamination produce obvious symptoms which can alert the researcher to a problem. Markers such as increased turbidity, drastic
changes in the pH color indicator included in many types of media, and floating colonies that are visible to the naked eye often present themselves well before significant cell death occurs. The suspect culture can then be properly disposed of before contamination can spread. While annoyance and wasted resources may result, contamination of this type is generally easy to identify and contain.

Mycoplasma contamination, on the other hand, is a different story. Mycoplasma, originally known as “pleuropneumonialike organisms” or PPLO, are tiny prokaryotes characterized by their lack of a rigid cell wall. Their presence in cultured cells was first described in 1956, when a group of researchers using HeLa cells to study PPLO discovered that their ostensible control cultures were already infected [281]. It has since been determined that mycoplasma are alarmingly widespread; studies have demonstrated that anywhere from 15 - 80% of cell cultures in use across the world are currently contaminated [282]. The reach of this problem can be attributed to several factors. First, the flexibility of mycoplasmal cell membranes in combination with their extremely small size allows them to pass through many biological filters commonly used to remove typical bacteria and fungi from reagents [282]. Secondly, the presence of mycoplasma in a culture does not always have an easily measurable impact. Depending upon the cell line, the type of mycoplasma, and the severity of the infection, cells may appear to grow normally and undergo little to no morphological change. Mycoplasma can also attain high densities in cell culture without inducing significant change in the turbidity or pH of the media [281]. They therefore have great potential to go undetected in labs that do not perform regular testing, all the while exerting a wide range of effects upon the cell cultures they infect, including alteration of cellular metabolism, induction or suppression of cytokine expression, induction of chromosomal aberrations, and alteration of signal transduction [282]. Results derived from experiments using mycoplasma-infected cultures are therefore unreliable at best and invalid at worst.
**Detection and Elimination of Mycoplasma Contamination.** The cryptic nature of many mycoplasma infections means that regular testing for this organism is the only way to ensure the validity of cell-culture-derived experimental results. Direct culturing of mycoplasma colonies on agar is the most sensitive testing technique; however, it presents major technical difficulties. First, it involves a complex culturing system involving both liquid and semisolid media as well as tightly-controlled conditions, including, ideally, an anaerobic environment [283]. It also requires the use of live mycoplasma as a control, thus necessitating the introduction of a known contamination source to the laboratory. Finally, as mycoplasma are relatively slow-growing, the entire process is quite time-consuming; it can take over a week for visible mycoplasma colonies to appear on a test plate [282], and the direct culture test in total can take up to 28 days [281]. These factors in combination are restrictive for most laboratories.

While commercial testing services do exist, many researchers turn instead to indirect methods, which detect specific characteristics of mycoplasma rather than culturing the organisms themselves. Mycoplasma detection kits based on PCR, enzyme assays, and ELISA are available, but DNA fluorochrome staining is the most popular and widely recommended indirect test as many laboratories already have the necessary materials and equipment on hand [281]. These fluorochrome stains are not specific for mycoplasmal DNA; rather, a mycoplasma infection will manifest as fluorescence that appears to have an extranuclear and/or extracellular localization (Figure A.1). Interpretation of the results is not always straightforward, as false positives may be caused by overstaining, debris on the slide, or high cell confluency. The use of commercially prepared positive control slides or photomicrographs for comparison is therefore recommended.

Considering the potentially high monetary and scientific costs of mycoplasma contamination, it is unsurprising that researchers who experience this setback often attempt to rescue the affected cell lines. A number of approaches for the elimination of mycoplasma from cell cultures have been developed, including physical procedures such as filtration, chemical treatments such as exposure to antibiotics or detergents, and immunological techniques such as *in*
Figure A.1. Mycoplasma contamination can be indirectly detected by DNA fluorochrome staining. Fluorescence photomicrograph of Vero indicator cells stained with Hoechst 33258. The large oval bodies are the Vero nuclei whereas the mycoplasma are seen as numerous points of extranuclear fluorescence in the background. From Hay, R. J., Macy, M. L., and Chen, T. R. Mycoplasma infection of cultured cells. Reprinted by permission from Macmillan Publishers Ltd: *Nature* 339:8, 487-488, ©1989.

*vivo* culture in nude mice [282]. However, not even the preferred approach, antibiotic treatment, is completely reliable; the three most effective antibiotics for mycoplasma contamination, Mycoplasma Removal Agent (MRA), coprofloxacin, and BM-Cyclin, have success rates of only 64%, 77%, and 84%, respectively [284]. Furthermore, the process of completely cleaning a mycoplasma-contaminated cell culture is difficult and time-consuming. Ideally, one would first identify which mycoplasma species are present so that proper antibiotics can be selected, then experimentally determine an antibiotic concentration that kills mycoplasma without inducing cytotoxicity in the culture. Several rounds of treatment are usually required, after which a minimum post-treatment antibiotic-free period of three to five weeks must be observed before resuming normal use of the cells [282]. Throughout this process, the continued presence of mycoplasma in the laboratory greatly increases the chance that other cell lines will become contaminated. The rescue of mycoplasma-infected cell lines thus is a risky undertaking with a significant probability of failure; it should be attempted only when the culture is irreplaceable. In most cases, the best way to deal with a mycoplasma-infected cell line is simply to autoclave it.

**Management of mycoplasma contamination during the course of this project.** While care was taken in our laboratory to maintain a clean workspace and practice good aseptic technique,
there was no regular mycoplasma testing program in place, as is the case in the majority of facilities worldwide [281]. Aberrant growth patterns in a line of NAM-treated IMR-90 cells during the course of this project raised concerns of possible mycoplasma infection. The culture in question, as well as all other cultures being used in the lab, were evaluated for the presence of mycoplasma. This process is described below.

**Experimental Methods**

**Materials and reagents.** Unless stated otherwise, reagents were purchased from either Sigma-Aldrich or Fisher Scientific. Cell culture reagents were purchased from Invitrogen, ATCC, or Atlanta Biologicals. Flasks and plates for cell culture were supplied by Corning. All cell lines were obtained from ATCC.

**Mammalian cell culture.** All cell manipulations were performed in a laminar flow hood under aseptic conditions. IMR-90 human lung fibroblasts and BJ human foreskin fibroblasts were grown in 75 or 150 cm$^2$ tissue culture flasks and maintained in a 37° C incubator at 5% CO$_2$ in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. 5 mM NAM prepared from a 1 M filter-sterilized stock solution was added as indicated. Media was replaced every 3-4 days. Cells were subcultured at 70-85% confluence by decanting the media, rinsing the flask with PBS, and adding 0.25% trypsin-EDTA (2-3 mL, prewarmed to 37° C). The cells were incubated at 37° C for 5-10 minutes until cell detachment occurred. Trypsin was neutralized by addition of supplemented DMEM to a final volume of 10 mL and cells were seeded in fresh flasks at a 1:3 to 1:8 ratio. During subculturing, a portion of cell suspension was combined with an equal volume of trypan blue live/dead stain, incubated for 1-2 minutes, and then counted using a hemocytometer to determine the total number of live and dead cells. Percent cell viability was calculated using the following formula, where $V = \text{percent viability}$, $S = \text{the number of stained cells}$, and $U = \text{the number of unstained}$
cells:

\[
V = \left( \frac{S}{S + U} \right) (100\%)
\]

Population doubling was calculated using the following formula, where \(H\) = the number of cells harvested, \(S\) = the number of cells seeded, and \(X\) = the number of PDs elapsed since the previous count:

\[
\log H - \log S = X(\log 2)
\]

Approximately every 5 PDs, several 2 mL aliquots of cells were prepared in supplemented DMEM containing 8% DMSO as a cryoprotectant. Cells were frozen overnight at -80° C, then stored under vapor phase nitrogen. To start cultures from frozen stock, cells were thawed quickly in a 37° C waterbath and transferred to a T-75 flask containing approximately 20 mL supplemented DMEM. Medium was replaced after 24 hours to remove cryoprotectant.

**DAPI staining.** IMR-90 and BJ fibroblasts were cultured on sterile coverslips resting in 6-well plates to 60-80% confluency. Media was removed and coverslips were washed twice briefly with 1 mL/well PBS. Cells were fixed in 2% formaldehyde/PBS solution for 5 min at room temperature, then washed twice for 5 min with 1 mL/well PBS. Approximately 300 µL of 300 nM DAPI (Invitrogen) in PBS was added to each coverslip and cells were incubated for 5 min at room temperature. The staining solution was aspirated and coverslips were washed twice briefly with 1 mL/well PBS. Coverslips were rinsed in deionized water, allowed to dry, and affixed to glass microscope slides using Prolong Gold (Invitrogen) according to the manufacturer's instructions. Images were obtained on a Deltavision deconvolution microscope equipped with a DAPI filter and a 100x oil immersion objective.

**Mycoplasma testing.** Mycoplasma testing was performed using Invitrogen’s MycoFluor reagent according to the manufacturer’s instructions. Briefly, IMR-90 and BJ fibroblasts were cultured on sterile coverslips resting in 6-well plates to 70-80% confluency. Fixative solution (3:1 v/v 100% methanol:glacial acetic acid) was added to the growth medium until the pH color in-
dicator changed from red to yellow (approximately 1 volume of fixative per volume of medium). Cells were fixed at room temperature for 5 min. Medium/fixative mixture was removed, fresh fixative solution was added to cover, and cells were fixed at room temperature for an additional 10 min. Cells were washed twice briefly with deionized water, after which volume per well was brought to 1.9 mL with deionized water. 100 μL of MycoFluor reagent was added to each fixed cell preparation. Coverslips were immediately removed from the staining solution and placed cell-side down on glass microscope slides, then sealed with melted wax and allowed to set. A sample of mycoplasma MORFS was also stained and mounted for comparison. Images were obtained on a Deltavision deconvolution microscope using the DAPI filter and 100x oil immersion objective.

Results and Discussion

Slow growth in nicotinamide-treated IMR-90 fibroblasts indicated possible contamination with mycoplasma. Though many cases of mycoplasma contamination do not produce obvious symptoms, it is not uncommon for infected cells to display aberrant growth patterns. It is important to keep careful record of cell growth so that changes may be identified. Accordingly, cell counts and population doubling totals were determined at every subculturing during the generation of cell stocks for this project, and a running plot of PDs vs. time for all cell lines (IMR-90 ±NAM and BJ ±NAM) was maintained. Examination of these plots revealed an unexplained change in the growth of NAM-treated IMR-90 cells. While they initially exhibited increased cell proliferation as expected, their growth rate decreased abruptly at about PD 43 and was soon surpassed by that of the untreated cells (Figure A.2). The NAM-treated IMR-90 growth curve began flattening out at between 40 and 50 days of culture, appearing similar to that of a senescing cell population. Untreated IMR-90 cells, however, continued growing robustly for several weeks past that point. As NAM is known to increase both cell proliferation and replicative lifespan in IMR-90 fibroblasts [191], this growth pattern was considered to be indicative of contamina-
Figure A.2. Nicotinamide-treated IMR-90 fibroblasts exhibited an aberrant growth pattern. Young IMR-90 fibroblasts were allowed to proliferate in the presence or absence of 5 mM NAM. Cells were subcultured and counted at 70-80% confluency and population doublings vs. time was plotted. While the NAM-treated cells initially exhibited accelerated proliferation as expected, their growth rate slowed abruptly at around PD 43 and was soon surpassed by that of the untreated cells.

The culture exhibited none of the visible changes commonly associated with bacterial or fungal contamination, and furthermore was being grown in the presence of conventional antibiotics (penicillin/streptomycin) and an antifungal (amphotericin B). Mycoplasma infection was therefore suspected.

**DAPI staining of nicotinamide-treated IMR-90 fibroblasts was indicative of mycoplasma contamination.** DNA fluorochrome staining was performed in order to further investigate possibility of mycoplasma contamination. The slow-growing NAM-treated IMR-90 cells showed multiple signs of mycoplasma infection as judged by DAPI staining, including widespread semi-punctate extranuclear fluorescence and the presence of filamentous structures (Figure A.3A). The filamentous structures in particular distinguished this staining pattern from that of ordinary DAPI background fluorescence due to overstaining or other technical issues. Simultaneous DAPI staining of the other normally-growing cell lines being cultured for this project showed little evidence of mycoplasma contamination. Extranuclear fluorescence was not observed in control IMR-90 cells (Figure A.3B), and the minor punctate background staining observed in NAM-treated BJ cells (Figure A.3C) appeared to result from slide debris. These findings in combination with cell growth records led to the conclusion that the NAM-
Figure A.3. DAPI staining of nicotinamide-treated IMR-90 fibroblasts was indicative of mycoplasma infection. IMR-90 and BJ fibroblasts were fixed and stained with DAPI. NAM-treated IMR-90 cells at PD 50 (A), which had been exhibiting an aberrant growth pattern, showed multiple signs of mycoplasma infection including severe semi-punctate extranuclear staining and the presence of filamentous structures (yellow arrows). These characteristics were not seen in normally growing IMR-90 cells (B) or NAM-treated BJ cells (C) (PD 54 and 55, respectively).

IMR-90 and BJ fibroblasts were fixed and stained with DAPI. NAM-treated IMR-90 cells at PD 50 (A), which had been exhibiting an aberrant growth pattern, showed multiple signs of mycoplasma infection including severe semi-punctate extranuclear staining and the presence of filamentous structures (yellow arrows). These characteristics were not seen in normally growing IMR-90 cells (B) or NAM-treated BJ cells (C) (PD 54 and 55, respectively).

treated IMR-90 cells were in fact contaminated with mycoplasma. This culture was consequently removed from the laboratory and autoclaved.

Mycoplasma testing with a commercial kit confirmed that all other cell lines were free of contamination. While cell growth records and the DAPI stain indicated that only the NAM-treated IMR-90 culture was contaminated, it was important to confirm this result by other means. We therefore tested the control IMR-90 cells, control BJ cells, and NAM-treated BJ cells using Invitrogen's MycoFluor detection kit. This kit relies upon a proprietary DNA fluorochrome preparation to achieve a level of sensitivity comparable to that of radioisotopic mycoplasma RNA detection [285]. The kit also includes mycoplasma MORFS (Microscopic Optical Replicas for Fluorescence assayS), which mimic the appearance of mycoplasma and can thus serve as a positive control, eliminating the need to bring infectious organisms into the lab.

MycoFluor staining of the mycoplasma MORFS supported our previous conclusion that the NAM-treated IMR-90 cells had been contaminated (Figure A.4A). Both the widespread, semi-punctate staining and the small, bright aggregations were similar in appearance to the patterns observed in DAPI images from that cell line. MycoFluor staining of control IMR-90 cells (Figure A.4B), control BJ cells (Figure A.4C), and NAM-treated BJ cells (Figure A.4D) showed
Figure A.4. Mycoplasma testing with a commercial kit confirmed that all other cell lines were free of contamination. IMR-90 and BJ fibroblasts were fixed and stained with Invitrogen’s MycoFluor reagent. MORFS provided by Invitrogen were also stained and mounted to provide an example of some typical presentations of various mycoplasma. (A) Mycoplasma MORFS. Both the diffuse staining and the small aggregations were similar in appearance to the DAPI stain of mycoplasma-infected NAM-treated IMR-90 cells (Figure A.3A). Contamination was absent in control IMR-90 cells (B), control BJ cells (C), and NAM-treated BJ cells (D) (PD 55, 52, and 58, respectively).

no evidence of mycoplasma contamination. In fact, all images of these cells were completely free of background fluorescence. These results in combination with normal growth patterns demonstrated by each of these lines allowed us to conclude that they were contamination-free, and to proceed normally with cell stock preparation.

Conclusion

This incident of mycoplasma contamination was less than catastrophic. The infection was contained to one batch of cells, and clean frozen stocks were on hand to reestablish the affected line. Nevertheless, the project was significantly set back by both the time-consuming nature of the testing process and the necessity of regrowing NAM-treated IMR-90 cells from PD 30 back up to PD 50 and then to their normal senescence point; this took several months. In addition, hundreds of dollars’ worth of cell culture materials and reagents were wasted. Despite eventual completion of the project, the cost of contamination is clear.

Preventative measures in place included the use of good aseptic technique and the maintenance of a clean workspace including a certified laminar flow hood, properly maintained incubators and waterbath, and prompt disposal of biological waste. Unfortunately, one intended safeguard — the supplementation of cell culture media with antibiotics — may
have actually contributed to the problem. While the routine use of antibiotics in cell culture is widespread, this practice is increasingly being discouraged as it may serve to mask mycoplasma contamination. First, though commonly used antibiotics such as penicillin and streptomycin are not very effective against mycoplasma, they can partially suppress a mycoplasma infection to the extent that no outward symptoms are observed, providing ample opportunity for the invading organism to spread to other cultures [282]. Secondly, mycoplasma contamination triggered by the generation of droplets or aerosols during cell culture work is likely to also include a mixture of bacteria and fungi. The use of antibiotics and/or antifungals in the media prevents the appearance of highly visible contamination which would otherwise develop within days of the contamination event, alerting a researcher to the problem and permitting quick disposal of the affected cultures [281]. For these reasons it is best to use antibiotics only as necessary on a temporary basis, e.g. during the creation of primary stocks and over the course of critical short-term experiments.

It is estimated that cell culture contamination by mycoplasma and other invasive organisms costs researchers millions of dollars annually in the US alone [281]. Even more troubling is the fact that it is impossible to know the extent to which published and commonly excepted experimental results should be questioned due to lack of mycoplasma testing in the contributing laboratory. While these problems will never be completely eliminated, widespread awareness of the issue, establishment of routine testing programs, and conservative use of antibiotics can greatly minimize their effects.
Bibliography


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