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Basal Activity of a PARP1-NuA4 Complex Varies Dramatically across Cancer Cell Lines

Graphical Abstract

Highlights

PARP1 activity varies strongly across cell lines independent of DNA strand breaks

PARP1 exists in at least three biochemically distinct states

Basal activity of a PARP1-NuA4 complex accounts for differences across cell lines

NuA4 is required for high basal activity of PARP1

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In Brief

Krukenberg et al. find that the enzymatic activity of PARP1 is highly variable across cancer cell lines. A complex containing PARP1 and the NuA4 chromatin-remodeling complex is responsible for the differences in PARP1 activity, and NuA4 subunits are required for high PARP1 activity. This work thus provides evidence for a mechanism of PARP1 activation and a direct link between PARP1 and chromatin remodeling outside of the DNA damage response.

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Basal Activity of a PARP1-NuA4 Complex Varies Dramatically across Cancer Cell Lines

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SUMMARY

Poly(ADP-ribose) polymerases (PARPs) catalyze poly(ADP-ribose) addition onto proteins, an important posttranslational modification involved in transcription, DNA damage repair, and stem cell identity. Previous studies established the activation of PARP1 in response to DNA damage, but little is known about PARP1 regulation outside of DNA repair. We developed an assay for measuring PARP activity in cell lysates and found that the basal activity of PARP1 was highly variable across breast cancer cell lines, independent of DNA damage. Sucrose gradient fractionation demonstrated that PARP1 existed in at least three biochemically distinct states in both high- and low-activity lines. A discovered complex containing the NuA4 chromatin-remodeling complex and PARP1 was responsible for high basal PARP1 activity, and NuA4 subunits were required for this activity. These findings present a pathway for PARP1 activation and a direct link between PARP1 and chromatin remodeling outside of the DNA damage response.

INTRODUCTION

Poly(ADP-ribose) (PAR) is a reversible posttranslational modification involved in multiple essential cellular processes including DNA damage, transcriptional control, and stem cell identity (Beneke, 2012; Chiou et al., 2013; Doege et al., 2012; Hassa and Hottiger, 2008; Ji and Tulin, 2010; Krishnakumar and Kraus, 2010a; Ogino et al., 2007; Tallis et al., 2014). Using nicotinamide adenine dinucleotide (NAD+) as a substrate, poly(ADP-ribose) polymerases (PARPs) polymerize ADP-ribose subunits onto acceptor proteins, forming large, negatively charged polymers of varying length (Schreiber et al., 2006; Tan et al., 2012). Polymers can be quickly hydrolyzed by poly(ADP-ribose) glycohydrolases (PARGs), leading to turnover of the NAD+ pool (Diefenbach and Bürkle, 2005; Hassa and Hottiger, 2008). Covalent attachment of PAR to a protein (PARylation) can alter its function. PARP1, for example, loses its PARP activity upon automodification (Ferro and Olivera, 1982; Zahradka and Ebisuazaki, 1982). Alternatively, PAR can serve as a scaffolding molecule, recruiting downstream PAR-binding effectors (Sousa et al., 2012). Seventeen putative PARPs have been identified in humans, based on sequence homology (Schreiber et al., 2006), but not all possess PARP activity (Kleine et al., 2008). PARP1, localized primarily to the nucleus, is the most abundant family member in humans (Vyas et al., 2013; Wang et al., 2012) and has been mainly examined in the context of base excision repair (Sousa et al., 2012). Recently PARP1 was implicated in other DNA repair pathways as well as in pathways outside of DNA repair such as transcription (Ji and Tulin, 2013; Krishnakumar and Kraus, 2010b) and stem cell identity (Chiou et al., 2013; Doege et al., 2012; Ogino et al., 2007). The details of its involvement in any of these pathways remain poorly understood.

There is much interest in the use of PARP inhibitors as cancer therapeutics. At least six phase III trials are ongoing or being planned for PARP1 inhibitors (Garber, 2013). These trials focus mainly on targeting cancers with defects in homologous recombination in an effort to exploit the hypothesis that PARP1 inhibition is synthetically lethal with other DNA repair defects (Farmer et al., 2005; Javle and Curtin, 2011). However, the role of PARP1 in DNA damage does not fully explain the efficacy of PARP inhibitors (Audeh et al., 2010; Garnett et al., 2012; Lord and Ashworth, 2013). To better understand the utility of PARP inhibitors in the clinic, we must better understand the function and regulation of PARPs in cancer, especially PARP1, the common target of all the clinical candidates.

Despite their clinical as well as basic biological importance, fundamental questions about the regulation and cellular functions of PARPs remain unanswered. To explore potential roles outside of the DNA damage response, we investigated basal PARP activity across breast cancer cell lines and found, unexpectedly, large variation due to differences in basal PARP1 activation states and not in gene expression or protein abundance. Our findings provide a pathway for PARP1 activation and suggest that PARP1 exists in different biochemical states both within a single cell line as well as between cell lines. Our findings further the basic understanding of PARP1 biochemistry and suggest roles for PARP1 outside of the DNA damage response.
RESULTS

Basal PARP1 Activity Varies Strongly across Breast Cancer Cell Lines

To profile basal activation states of PARP, we measured PARP activity in cell lysates, in the absence of DNA damage, across a panel of breast-cancer-derived cell lines. We used a bead-based capture assay optimized for lysate measurements that allowed for better quantification of PAR levels than the standard immunoblot-based assay. Our assay is complimentary to a recent mass spectrometry method quantifying steady-state PAR levels in cells or tissues (Martello et al., 2013). Lysates were prepared from cells grown under standard, nonstressed growth conditions. A PARG inhibitor, ADP-HPD, was added to the lysis buffer to prevent degradation of PAR during the assay, which was important because PAR degraded quickly in its absence. PAR was captured onto beads coated with an anti-PAR monoclonal antibody and detected using a tandem zinc-finger PAR-binding domain from the protein APLF (Ahel et al., 2008). PAR, produced and purified in vitro (Tan et al., 2012), was used as a standard. Addition of a PARP inhibitor directly to the lysis buffer confirmed that the assay measures PAR accumulation in lysates, but not preformed PAR from cells, because most of the detectable PAR accumulated after lysate preparation (Figure S1). Thus, the assay measures total PARP activity in lysate, not cellular PAR levels.

We found a surprising degree of variation in total PARP activity across 11 breast cancer cell line lysates, with up to 60-fold variation, using the assay (Figure 1A). Differences in polymer levels were also seen by immunoblot for PAR, a standard assay in the field (Figure S1). We were unable to detect a PARP-dependent signal by immunofluorescence (data not shown), confirming that our assay measures accumulation of PAR in the lysates and not basal levels. We further validated this result using a radioactive polymerization assay. Cell lysates from MCF7 (high activity) or T47D (low activity) cells were incubated with 32P-NAD+ in the presence of ADP-HPD, and the rate of PAR synthesis was measured using a filter-binding assay. Synthesis rates were much higher in MCF7 cell lysates (Figure 1B). The rate of PAR degradation was measured by adding preformed 32P-labeled PAR to lysates in the presence of ABT-888 to block further synthesis. MCF7 and T47D cell lysates showed similar rates of PAR degradation (Figure 1C). We concluded that the basal activity of one or more PARPs is differentially regulated between MCF7 and T47D cells, whereas the rates of PAR degradation do not vary significantly in the two lines tested.

To determine which PARP family members are activated in MCF7 cells, we measured PARP activity in cell lysates from cells treated either with the PARP1–PARP4 inhibitor ABT-888 or the PARP5a/PARP5b inhibitor XAV939 (Wahlberg et al., 2012). XAV939 also binds PARP1/PARP2 but with approximately 10-fold lower affinity than to PARP5a/PARP5b (Huang et al., 2009a; Wahlberg et al., 2012). Across the cell lines tested, PARP activity was reduced by >97% in the presence of ABT-888, whereas XAV939 reduced PARP activity by 30%–70% (Figure 2A). We further narrowed the possibilities with a PARP1/PARP2-specific inhibitor, niraparib (Jones et al., 2009), which reduced activity by ~95% (Figure S2A). Of PARP1 and PARP2, PARP1, due to its high abundance, was the most obvious candidate. Using small interfering RNA (siRNA), we knocked down PARP1 in two cell lines with high PARP activity, KPL-1 and MCF7. In both cell lines, PARP1 knockdown of 70%–90% (Figure 2B) resulted in an approximately 80% loss in lysate PARP activity (Figure 2C). We confirmed by RT-PCR that PARP1 siRNA was specific to PARP1 and did not affect PARP2 RNA levels (Figure S2B). These data indicate that PARP1 is the primary enzyme responsible for the high basal PARP activity in the tested cell lines.
PARP1 activity could be regulated at either the mRNA or protein level. We compared PARP1 transcript levels across cell lines using published data (Neve et al., 2006). The small differences in transcript levels found seemed unlikely to account for the activity differences (data not shown). We then measured protein levels using immunoblots. Indeed, PARP1 protein levels were relatively constant across cell lines (Figure 2D), and the small differences observed did not correlate with differences in PARP1 activity. Instead, the results are consistent with the enzymatic activity of PARP1 being regulated through mechanisms other than transcription, translation, or degradation.

PARP1 Activation Is Independent of DNA Strand Breaks

Based on the literature, our initial hypothesis was that PARP1 is basally activated in some cancer cell lines due to spontaneous DNA damage. To test this hypothesis, we measured DNA damage using phospho-histone H2Ax, which forms foci at the sights of DNA strand breaks, as a marker. Immunofluorescence measurements of phospho-H2Ax in fixed cells, grown under standard conditions in the absence of DNA-damaging agents, showed no significant correlation between basal phospho-H2Ax staining and basal PARP activity was found (Figures S3A–S3C). We also measured the levels of phospho-ATM, ATR, Chk1, and Chk2 as other markers for activation of the DNA damage-repair pathways. We saw very little activation of these proteins in the absence of a DNA-damaging agent (Figure S3D). We concluded that spontaneous DNA damage does not explain the differences in basal activation of PARP1 in these cell lines.

Another possible explanation is that DNA was released during lysate preparation and its presence activated PARP1 in lysates. To test this, we blotted for histones in either the lysate supernatant or pellet. We found 70%–80% of total PARP1 in the supernatant and no histone H4. Histone H4 was only found in the pellet (Figure S4A), indicating that the active PARP1 we were measuring was nucleoplasmic and not tightly bound to chromatin. It also suggested that no or very little chromatin was present in our lysate preparations. To further test for the presence of DNA, the DNA-binding dye YOYO-1 was added to control lysates and lysates treated with DNase I. YOYO-1 fluoresces upon binding to nucleic acids and detects picograms of DNA. We observed weak signal in untreated lysates corresponding to 1 to 2 pg of nucleic acid/ng of total protein (0.1% of the total protein). This signal was the same for both MCF7 and T47D lysates. The signal remained after DNase I treatment of the lysates (Figure S4B). We also ran precleared and cleared lysate samples treated with RNase on agarose gels and stained for nucleic acids with SYBR Safe (Invitrogen; Figure S4C). We saw no staining in the cleared lysate samples, indicating that DNA was removed from the lysates upon centrifugation. Based on the limits of detection in our YOYO-1 assay, these experiments show that there is less than 0.02 pg of DNA/ng of protein in the lysate. The DNase-resistant YOYO-1 signal may result from binding to RNA in the lysates. Even if a small amount of DNA is present, it appears to be the same for the two samples and cannot account for the differences in PARP activity.
PARP1 Is Found in Multiple Biochemically Distinct Complexes

As an unbiased approach to characterizing cell line differences, we fractionated cell lysates using sucrose gradients and measured PARP activity and PARP1 protein levels across fractions. The PARP activity of each fraction was measured in the presence and absence of ABT-888 to confirm specificity of NAD\(^+\) incorporation (Figures 3A and 3B). The distribution of PARP1 protein between fractions was similar in both high- and low-activity lines (Figures 3C and 3D). Approximately 44% was found in a fraction whose mobility was that expected of uncomplexed PARP1 sedimenting as a monomer or dimer. The sucrose gradient cannot distinguish between a monomer and a dimer, but previous studies have shown that PARP1 functions as a dimer (Mendoza-Alvarez and Alvarez-Gonzalez, 1993; Pion et al., 2005). Approximately 10% of PARP1 protein was found in an ~700 kD region and ~35% in a high-molecular-weight region (~1 MD). The distribution of PARP1 protein was similar in both cell lines.

Basal PARP1 activity was detected by \(^{32}\)P-NAD\(^+\) polymerization. Activity in the uncomplexed PARP1 was undetectable. In MCF7 (high activity) lysates, PARP1 activity was strong in the 700 kD and >1 MD fractions. We compared activity to protein levels measured by immunoblot to estimate specific activity in arbitrary units and found the highest specific activity in the ~700 kD fraction (Figures 3E and 3F). This fraction was responsible for most (~75%) of the increased basal PARP1 activity in MCF7 cells as compared to T47D cells.

To determine if PARP1 activity could be increased, we added nicked DNA to each fraction and remeasured the PARP1 activity (Figures 3G and 3H). We found that even the most active PARP1-containing fractions could be further activated with the addition of nicked DNA. Both the 700 kD and >1 MD fractions were activated to approximately the same level of specific activity. The uncomplexed fraction showed some increase in activity but was the least activated in the presence of DNA. Taken together, the fractionation data indicate that PARP1 exists in at least three separate forms, each with a distinct basal activation state and potential for activation by nicked DNA. The difference in PARP activity between the two cell lines derives mainly from different basal activation states of the 700 kD fraction.

PARP1 with High Basal Activity Is in Complex with NuA4

To find candidate binding partners, fractions 2+3, 6, and 9+10 from an MCF7 lysate sucrose gradient were immunoprecipitated with anti-PARP1, and coprecipitating proteins were identified via tandem mass spectrometry of tryptic peptides (liquid chromatography-tandem mass spectrometry [LC-MS/MS]). Spectral counts of PARP1 peptides across the three fractions correlated well with the abundance of PARP1 protein measured by immunoblotting (Tables 1 and S1; Figure 3C), showing quantitative recovery of PARP1 protein. We detected no significant binding partners in the uncomplexed fraction. We were particularly interested in fraction 6, given its high basal PARP activity in MCF7 (high activity) cells as compared to T47D (low activity) cells. Enrichment analysis was performed on the top 50
proteins immunoprecipitated from fraction 6 using the DAVID bioinformatics platform (Huang et al., 2009b). The top enrichment group consisted of eight members of the 16-member NuA4 chromatin-modifying complex; this enrichment was also obvious from visual inspection of the data. Further analysis of fraction 6 LC-MS/MS data identified 14 subunits of the NuA4 complex that were highly enriched for fraction 6 when compared to fractions 2+3 and fractions 9+10 (Tables 1 and S1).

To test the hypothesis that NuA4 binding correlates with PARP1 activity, relative levels of PARP1-binding partners were compared between MCF7 total lysates and T47D total lysates using stable isotope labeling by amino acids in culture (SILAC) in combination with LC-MS/MS. PARP1 was immunoprecipitated from MCF7 and T47D lysates after growing one cell line in heavy amino acids, allowing for the relative quantification of proteins identified in both cell lines. Nine subunits of the NuA4 complex were enriched in MCF7 PARP1 immunoprecipitations from two biological repeats (Table 1). Thus, more NuA4 was in complex with PARP1 in the high-activity line. These data are consistent with a hypothesis that binding to NuA4 activates PARP1 in the high-activity line.

To retest the PARP1-NuA4 interaction, reciprocal immunoprecipitations were performed by immunoprecipitating either the EP400 or KAT5(Tip60) subunits of the NuA4 complex and measuring PARP1 activity (Figure 4A). PARP1 coimmunoprecipitated with both NuA4 subunits in both MCF7 and T47D cell lysates. A small amount of PARP1 nonspecifically bound to immunoglobulin G (IgG), but levels of PARP1 in the EP400 and KAT5 immunoprecipitations were higher than in the IgG control. In the reverse, EP400 coimmunoprecipitated with both PARP1 and KAT5. A small amount of KAT5 may coimmunoprecipitate with PARP1 and EP400, but it was difficult to measure due to high background in the immunoblot. Next, we measured PARP1 activity (via 32P-NAD+ incorporation) and KAT5 activity (via in vitro acetylation of histone H4) in each immunoprecipitated complex from MCF7 cell lysates (dark bars) and T47D cell lysates (light bars). Representative data from three independent experiments is shown. See also Figure S5.

Table 1. Active PARP1 Coimmunoprecipitates with Components of the NuA4 Complex

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>2+3</th>
<th>6</th>
<th>9+10</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP1</td>
<td>147</td>
<td>27</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>TRRAP</td>
<td>93</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP400</td>
<td>6</td>
<td>69</td>
<td>4</td>
<td>0.63 ± 0.34</td>
</tr>
<tr>
<td>RUVBL1</td>
<td>31</td>
<td>7</td>
<td></td>
<td>0.64 ± 0.074</td>
</tr>
<tr>
<td>RUVBL2</td>
<td>28</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPC1</td>
<td>19</td>
<td></td>
<td></td>
<td>1.9 ± 0.17</td>
</tr>
<tr>
<td>ACTL6A</td>
<td>13</td>
<td>4</td>
<td></td>
<td>1.1 ± 0.092</td>
</tr>
<tr>
<td>DMAP1</td>
<td>13</td>
<td>1</td>
<td></td>
<td>1.2 ± 0.15</td>
</tr>
<tr>
<td>BRD8</td>
<td>10</td>
<td>1</td>
<td></td>
<td>0.93 ± 0.42</td>
</tr>
<tr>
<td>YEATS4</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ING3</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPS72</td>
<td>5</td>
<td>1</td>
<td></td>
<td>0.81 ± 0.25</td>
</tr>
<tr>
<td>KAT5</td>
<td>3</td>
<td></td>
<td></td>
<td>1.8 ± 0.18</td>
</tr>
<tr>
<td>MORF4L1</td>
<td>2</td>
<td>1</td>
<td></td>
<td>1.1 ± 0.16</td>
</tr>
<tr>
<td>MORF4L2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PARP1 was immunoprecipitated from the indicated sucrose gradient fractions (sucrose gradient) or total lysates (SILAC). For SILAC, one cell line was grown with heavy amino acids to facilitate quantification of the relative abundance of peptides between the two cell lysates. Spectral counts, a measure of relative abundance, are the number of MS2 spectra for a given protein. SILAC ratios, relative protein abundance between the two cell lines, were normalized to the ratio for PARP1, the natural log (ln) of each ratio was taken, and the results of two biological repeats were averaged. The ln(ratios) for NuA4 proteins found in both repeats are shown. See also Table S1.

Figure 4. PARP1 Forms Two Biochemically Distinct Complexes with NuA4

(A) Immunoprecipitations were performed from 1 mg of total protein with the indicated antibodies. Immunoprecipitated proteins were probed for PARP1, EP400, or KAT5 by immunoblotting. The input sample contains 15 µg of total protein.

(B and C) PARP1 activity (B), via 32P-NAD+ incorporation, and KAT5 activity (C), via in vitro acetylation of histone H4, were measured in each immunoprecipitated complex from MCF7 cell lysates (dark bars) and T47D cell lysates (light bars). Representative data from three independent experiments is shown. See also Figure S5.
These data indicate that NuA4 binds either active (from MCF7 cells) or inactive (from T47D cells) PARP1.

To test if active KAT5 was part of the active PARP1-NuA4 complex, we measured acetyltransferase activity in the PARP1, EP400, and KAT5 immunoprecipitations. Neither immunoprecipitated PARP1 or EP400 from MCF7 had acetyltransferase activity above the IgG control. Immunoprecipitated PARP1 and EP400 from T47D cells showed a small amount of activity. In both cell lines, immunoprecipitated KAT5 displayed high acetyltransferase activity (Figure 4C). These data indicate that KAT5 activity is not required for PARP1 activation in MCF7 cells.

To test if the NuA4-PARP1 complex is specific to cancer cells, we repeated the immunoprecipitation and activity experiments in retinal pigment epithelium (RPE) cells. RPE cells are immortalized, noncancer cells. They had low basal PARP activity, comparable to T47D cells (Figure S5A). In RPE cell lysates, PARP1 with low activity immunoprecipitated with both EP400 and KAT5, and the acetyltransferase activity of the PARP1 and EP400 immunoprecipitations was at the limit of detection (Figures S5B–S5D). Together with our previous results, we concluded that the PARP1-NuA4 complex is conserved across cancer and noncancer cell lines; however, the level of PARP1 activity within the complex differs between cell lines with higher activity observed in some cancer cell lines such as MCF7.

**Functional Consequences of the PARP1-NuA4 Interaction**

Whereas acetylation was not essential for PARP1 activation, PARP1 activity may affect histone acetylation by KAT5. We measured the acetylation levels of a known target of KAT5, histone H4 (Doyon and Côté, 2004). We treated cells for up to 72 hr with the PARP1/PARP2 inhibitor niraparib and then blotted for H4 acetylation at the K5, K8, or K12 positions (Figure 5A). No differences were seen for T47D. For MCF7, a small but significant decrease in acetylation of K5 was seen over 72 hr (Figure 5B).
NuA4 is also involved in gene regulation (Ginsburg et al., 2009; Yamada, 2012), and we hypothesized that high basal PARP1 activity of a PARP1-NuA4 complex affects transcription. We therefore measured global transcript levels via microarray in the presence and absence of PARP inhibitors. First, we compared MCF7 cells treated for 6 hr with DMSO, ABT-888, or olaparib, two chemically distinct PARP1 inhibitors. Remarkably, given the abundance and high basal activity of PARP1 in MCF7 cells, we found no significant differences in transcript levels (Figure S6), suggesting that the PARP1-NuA4 complex is not a direct regulator of transcription. To test for later transcriptional effects, we repeated the microarray experiment with cells treated with either ABT-888 or DMSO for 48 hr. We compared six cell lines, three with high PARP activity (MCF7, KPL-1, and ZR7530) and three with low PARP activity (T47D, SKBR3, and Hs578T). Cell-line-specific differences dominated the differences in transcription, and from a global perspective, PARP inhibitor treatment caused little change in transcript levels as compared to cell line differences. Unsupervised clustering placed drug-treated and untreated next to each other for all lines (Figure 5C). These data show that PARP1 inhibitors are not strong regulators of global transcription.

For a closer look at drug-induced changes, we binned the data into two groups, high PARP activity and low PARP activity, took the mean of each group, and ranked the genes by the difference of the means (Table S2). Our top hit, E2F1, is a transcription factor involved in cell cycle progression that has been implicated in apoptosis and as a tumor suppressor (Wang et al., 1999). E2F1 showed an increase in transcript levels in low-PARP-activity cell lines and a decrease in high-activity cell lines following drug treatment (Figure 5D). We next examined E2F1 protein levels in MCF7 and T47D cells after 24, 48, and 72 hr of niraparib treatment (Figure 5E). In both cell lines, there was an initial increase in E2F1 protein followed by a gradual decline. T47D cells (low PARP activity) showed consistently higher levels of E2F1 than MCF7 cells, consistent with RNA levels. At 72 hr of drug treatment, E2F1 protein level in T47D cells was equivalent to the untreated level, whereas the level in MCF7 cells was lower than the untreated level. We saw similar results in two additional cell lines: KPL-1 (high activity) and SKBR3 (low activity; Figure S6). These data indicate a role for PARP activity in regulating both RNA and protein levels for E2F1, and the magnitude of this regulation is different between the high- and low-PARP-activity cell lines.

Chromatin-Remodeling Subunits of NuA4 Are Required for High Basal PARP1 Activity

To test the hypothesis that NuA4 is required for activation of PARP1, we knocked down multiple subunits of NuA4 in MCF7 cells and measured basal PARP1 activity in lysates. Using siRNA, we knocked down the EP400, KAT5, RUVBL1, and DMAP1 subunits of NuA4. Knockdown of EP400 showed the most dramatic effect on PARP1 activity (Figure 6), reducing PARP1 activity to a similar extent as knockdown of PARP1 itself. RUVBL1 and DMAP1 knockdown also showed a reproducible decrease in PARP1 activity, although not to the same low levels as EP400 knockdown (Figure 6). This might be explained by partial knockdown of the siRNA target. Knockdown of KAT5 had no significant effect on basal PARP1 activity in MCF7 cells. Loss of PARP activity in the EP400, RUVBL1, and DMAP1 knockdowns could be explained by a concomitant loss of PARP1. We examined PARP1 protein levels in all knockdowns and found that NuA4 knockdowns did not significantly affect PARP1 levels (data not shown). Lack of an effect of KAT5 knockdown is consistent with the immunoprecipitation data, suggesting that acetyltransferase activity is not required to activate
PARP1. Rather, subunits of NuA4 involved in chromatin remodeling, EP400, RUVBL1, and DMAP1 (Yamada, 2012), are required for high basal PARP1 activity in MCF7 cells.

**DISCUSSION**

We found that PARP1 in cell lysate was distributed between at least three complexes differing in native molecular weight and interacting proteins. A low-molecular-weight fraction, which comprised almost half of the total PARP1, moved on sucrose gradients consistent with the expected mass of uncomplexed PARP1. This fraction was basally inactive by our means of detection and, interestingly, was not activated by nicked DNA, unlike bacteria-expressed PARP1. We speculated that this uncomplexed fraction was somehow modified to prevent its activation by nicked DNA. Two larger-molecular-weight pools of PARP1 (approximately 700 kD and >1 MD) contained most of the basal and DNA-inducible activity. The >1 MD fraction is the least well understood. It contained ~35% of the PARP1 polypeptide and had low levels of basal-specific activity but was highly inducible by nicked DNA. Mass spectrometry has not yet provided a consistent profile of PARP1-interacting proteins in this fraction, which is clearly worthy of further study. We concentrated our efforts on characterizing the fraction around 700 kD, which had the highest basal activity per unit protein, the largest difference between cell lines, and appeared to consist in part of a defined complex between PARP1 and NuA4. The components of the NuA4 complex have been previously identified in HeLa cells, but PARP1 was not identified (Doyon et al., 2004). Based on our results, PARP1 appears to be a dynamic member of the NuA4 complex, whose interaction was likely disrupted by the higher salt concentrations used in the HeLa cell purification.

Our primary focus was to understand the 10- to 60-fold differences in basal PARP1 activity found between breast cancer cell lines. Surprisingly, the distribution of PARP1 protein on sucrose gradients was similar between a high-activity cell line, MCF7, and a low-activity cell line, T47D. The intermediate-sized fraction, where PARP1 is complexed to NuA4, was hard to detect by PARP1 immunoblot alone, but immunoprecipitation data showed that this complex was present in both lines. Our data so far showed that the distribution of PARP1 between complexes was similar between cell lines and that the difference was restricted to strong activation of PARP1 in MCF7 cells and minimal activation (or possibly repression of activity) in T47D. This differential activation was strongest in the NuA4 complex. This complex was not limited to cancer cells and could be found in a noncancer cell line, RPE, where it had biochemical characteristics similar to the low-activity complex found in T47D cells.

Having identified the PARP1-NuA4 interaction, we began to investigate the mechanism of PARP1 activation. Through siRNA studies, we found that chromatin-remodeling components of NuA4 were required for the activation of PARP1. However, because NuA4 bound PARP1 in both high- and low-activity cell lines, binding alone, though necessary, was not sufficient for activation. In Drosophila, KAT5 is required for activation of PARP1 upon heat shock (Petesch and Lis, 2012). Our experiments indicate that KAT5 activity is not necessary for PARP1 activation. The data suggest that levels of NuA4, in particular EP400, may determine PARP1 activity. We examined transcript levels of EP400 across cell lines and found no correlation with PARP activity (Figure S7). Also, we saw no significant differences in EP400 protein levels between MCF7 and T47D cell lysates, and the amount of PARP1 that coimmunoprecipitates with EP400 was similar between the two cell lines. Instead of the amount or stoichiometry of the complex determining PARP1 activity, other enzymatic activities of NuA4 such as the ATPase activity of EP400 may be required, or NuA4 may facilitate the interaction of PARP1 with other activating factors. We are continuing to explore the mechanistic details of PARP1 activation in these cell lines.

Though KAT5 activity was not required for PARP1 activation, PARP1 may influence the histone acetylation functions of KAT5. We saw a small effect of PARP1 activity on the levels of acetylated histone H4(K5) in a high-PARP-activity cell line and no effect in a low-activity cell line, suggesting that PARP1 may play a role in regulating histone acetylation. Whereas our data are consistent with PARP1-regulating histone acetylation through the NuA4 complex, we cannot rule out the involvement of other acetyltransferases. H4(K5) acetylation is not specific to NuA4, and other acetyltransferases could modify this position.

Both PARP1 and NuA4 have reported roles in transcription (Doyon and Côté, 2004; Ji and Tulin, 2010; Krishnakumar and Kraus, 2010a). We found no significant effect on transcription after 6 hr of PARP inhibitor treatment, indicating that the PARP1-NuA4 complex does not directly regulate transcription. Surprisingly, we also found no strong global effect on transcription after 48 hr of PARP inhibitor treatment. One possibility is that high PARP1 activity primes cells to respond to other signals and the effects of PARP1 inhibition on transcription would be larger in the presence of another stimulus, as was seen in the case of prostate cancer cells treated with testosterone (Schiewer et al., 2012). By looking across multiple cell lines, we found PARP-dependent regulation of E2F1. This is consistent with a previous study showing PARP1 regulation of E2F1 transcription in fibroblasts (Similian-Rosenthal et al., 2003), and we confirmed regulation at the protein level. E2F1 is an important transcription factor with potential roles as a tumor suppressor, and this regulatory interaction may be one reason why some cancers upregulate PARP1 activity.

We undertook a comparative analysis of breast cancer cell lines in part to understand differential responses to PARP1 inhibitors in patients. We uncovered biochemistry of PARP1 suggesting that PARP1 inhibitors may work through mechanisms other than the currently proposed mechanisms of DNA repair. Cell lines with both high and low basal PARP1 activity were similarly unaffected by PARP1 inhibitors in culture, at least using standard cytotoxicity assays (not shown). However, high basal PARP activity might increase sensitivity to other drugs or combinations with PARP inhibitors.

Finally, our results highlight the importance of looking at differences in enzymatic activity when comparing cell lines. Many large-scale studies focus on transcript level differences between cancer cell lines, but as our study highlights, potentially important activity differences could be overlooked. Our study of basal differences in PARP1 activation highlights a functional interaction for PARP1 with a chromatin-remodeling complex and paves
the way for further elucidating non-stress-induced roles of PARP1 in the nucleus and the impact of those roles on cancer cell biology.

EXPERIMENTAL PROCEDURES

Cell Culture and Lysis

All cell lines (American Type Culture Collection) except KPL-1 and RPE were grown in RPMI (Mediatech/Corning) supplemented with 10% fetal bovine serum (FBS) (GIBCO). KPL-1 and RPE were grown in Dulbecco’s modified Eagle’s medium/F12 (Mediatech/Corning) supplemented with 10% FBS. For SILAC, cells were grown in RPMI without lysine or arginine (Caisson Labs) supplemented with 10% FBS and either light (12C, 14N) or heavy (13C, 15N) arginine and lysine (Sigma-Aldrich or Pierce). Cells harvested via trypsinization were washed with PBS and frozen at –80°C. Upon thawing, cells were lysed in lysis buffer (50 mM Tris [pH 8], 150 mM NaCl, and 1% NP-40) for 30 min on ice. Cell debris was pelleted at 4°C and 21,000 × g for 30 min. Lysate protein concentration was determined with the Bio-Rad DC protein assay. For acetylation, EZ21, and DNA damage marker immunoblots, cells were lysed directly in the tissue culture dish with 1× SDS loading buffer. Lysate was collected and sonicated before boiling and loading the gel.

Antibodies

For immunoblotting, the following antibodies were used: anti-PARP1 (Tulip Biolabs; 1051), anti-histone H4 and anti-acetyl-histone H4 (Lys5, Lys8, or Lys12; Cell Signaling Technology; 8346), monoclonal anti-tubulin clone DM1A (Sigma–Aldrich), monoclonal anti-actin clone AC-74 (Sigma–Aldrich), anti-phospho ATM, ATR, CHK1, and CHK2 (Cell Signaling Technology; 9947), anti-DMA1 (Bethyl Laboratories; A300-218A), anti-Tip60 (Calbiochem; DR1041), and anti-rabbit HRP (GE Healthcare). Proteins were visualized using the ECL 2 kit (Thermo Scientific) for HRP detection or the Odyssey (LI-COR Biosciences) for far-red (Dylight) fluorescent detection. For immunoprecipitation, the following antibodies were used: anti-PARP (Cell Signaling Technology; 46D11), anti-Tip60 (KATS) (Image; IMG-6313A), anti-rabbit Dylight 800 (Thermo Scientific), anti-mouse Dylight 880 (Thermo Scientific), anti-chicken IgY Dylight 800 (Thermo Scientific), anti-chicken IgY horseradish peroxidase (HRP; Abcam), anti-mouse HRP (GE Healthcare), and anti-rabbit HRP (GE Healthcare). Proteins were visualized using the ECL 2 kit (Thermo Scientific) for HRP detection or the Odyssey (LI-COR Biosciences) for far-red (Dylight) fluorescent detection. For immunoprecipitation, the following antibodies were used: anti-PARP (Cell Signaling Technology; 46D11), anti-Tip60 (KATS) (Image; IMG-6313A), anti-rabbit Dylight 800 (Thermo Scientific), anti-mouse Dylight 880 (Thermo Scientific), anti-chicken IgY horseradish peroxidase (HRP; Abcam), anti-mouse HRP (GE Healthcare), and anti-rabbit HRP (GE Healthcare). Proteins were visualized using the ECL 2 kit (Thermo Scientific) for HRP detection or the Odyssey (LI-COR Biosciences) for far-red (Dylight) fluorescent detection.

Protein Purification

The tandem zinc finger domain of human APLF (residues 376–450; APLF-PAR) was cloned into pET28a (Invitrogen) and expressed in E. coli strain BL21 (DE3) Rosetta pLysS (Novagen). Upon induction with isopropl β-D-1-thiogalactopyranoside, 500 µM ZnSO4 was added to the media. Protein was expressed overnight at 16°C. Cells were resuspended in 50 mM Tris (pH 7.4), 1 M NaCl, 150 µM ZnSO4, 10% glycerol, 1% Tween, 5 mM β-mercaptoethanol, and complete protease inhibitor cocktail (Roche) and lysed using a cell disruptor. Cell debris was pelleted for 30 min at 138,000 × g, and the supernatant was incubated with Ni-NTA resin (Qiagen) at 4°C for 2 hr. The nickel resin was washed with 20 mM Tris (pH 7.4), 1 M NaCl, 150 µM ZnSO4, 5 mM β-mercaptoethanol, and 20 mM imidazole and eluted with 20 mM Tris (pH 7.4), 200 mM NaCl, 150 µM ZnSO4, 5 mM β-mercaptoethanol, and 300 mM imidazole. APLF-PBD was then gel filtered on a Sephacryl S-100 column (GE Healthcare) using 50 mM Tris (pH 7.4), 200 mM NaCl, 100 µM ZnSO4, and 5 mM β-mercaptoethanol.

PARP1 and the catalytic fragment of PARG were purified as previously described (Tan et al., 2012).

PAR Detection in Cellular Lysates

Monoclonal PAR antibody 10H (Tulip Biolabs) was conjugated to carboxylated MagPlex beads (Luminex/Bio-rad). Briefly, 5 × 106 beads/ml were washed and resuspended in coupling buffer (50 mM monobasic sodium phosphate [pH 5.0], 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (Oakwood Products) was added to a final concentration of 5 mg/ml and incubated with the beads at room temperature for 20 min. Beads were washed in coupling buffer, resuspended with 8 µg antibody in coupling buffer, and incubated for 2 hr at room temperature. Conjugated beads (MagPlex-10H) were washed and stored at 4°C in PBS with 0.02% Tween, 0.1% BSA, and 0.05% azide.

To biotinylate APLF-PBD, 1.7 mg/ml purified protein was incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Invitrogen) for 1 hr at room temperature followed by 1 hr at 4°C. The reaction was quenched with 100 mM glycine.

For PAR detection, all lysates contained 1 µM ADP-HPD (Axoxa; EMD Biosciences). All dilutions and washes were done with PBS + 1% BSA, MagPlex-10H beads (50 beads/µl) and lysate (50–200 ng total protein/µl) were incubated together overnight at 4°C. Beads were then washed, resuspended in 3 ng/µl biotin-APLF-PBD, and incubated for 2 hr at room temperature. Next, beads were washed, resuspended in 2 ng/µl conjugated streptavidin-R-phycocerythrin (Thermo Scientific), and incubated at room temperature for 30 min. Finally, beads were washed, resuspended to a final concentration of 50 beads/µl, and analyzed on the FlexMap3D (Luminex). Free PAR, purified as previously described (Tan et al., 2012), was used as a standard for quantification.

32P PARP and PARG Activity Assays

For PARP activity, lysates (0.5–1 mg total protein/ml) were incubated with 5 µCi 32P-NAD+ (PerkinElmer; 800 Ci/mmol 5 µCi/ml) and 1 µM ADP-HPD in PBS or lysis buffer at 30°C. Reactions were quenched by adding trichloroacetic acid (TCA; Sigma–Aldrich) to a final concentration of 10%. Quenched reactions were filtered through nitrocellulose, precipitated in PBS, and washed with 1% TCA. 32P incorporation was quantified using a phosphor storage screen and scanner (Bio-Rad). Ten micromolar ABT-888 (Selleck Chemicals) was added as a control for PARG activity.

For PARG activity assays, 32P-labeled PAR-PARP1 was made through an automodification reaction as described previously (Tan et al., 2012). Lysate (0.5–1 mg total protein/ml) and 10 µM ABT-888 were added to the 32P-PARP-PARP1 in PBS. The reactions were quenched and analyzed as the 32P PARP activity assay.

Immunoprecipitation

Whole-cell lysates or sucrose gradient fractions containing a protease inhibitor cocktail (Roche) were incubated with antibody for 2 hr at 4°C. Prewashed Anti-Prep Protein A beads (Bio-Rad) were added and incubated for 1 hr at 4°C with the antibody/antigen complex. Beads were washed with 50 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EGTA, and 1 mM MgCl2. SILAC samples were washed with 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EGTA, and 1 mM MgCl2. For SDS-PAGE analysis, proteins were eluted from the beads by boiling in sample buffer, and immunoblots were performed using the LI-COR Biosciences Quick Western Detection Kit. Activity assays were performed directly on beads.

Sucrose Gradient Fractionation

Sucrose gradients (5%/40% w/v) were poured as step gradients of five equal volume steps in 50 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EGTA, and 1 mM MgCl2 and allowed to diffuse overnight into continuous gradients. Gradients were spun at 4°C and 237,000 × g for 4 hr in an SW55Ti rotor (Beckman Coulter Genomics). Gradients were fractionated from the top by pipetting. A standard containing thyroglobulin, catalase, aldolase, and BSA was run in parallel.

LC-MS/MS Analysis

PARP1 immunoprecipitation samples from SILAC experiments were reduced in 20 mM dithiothreitol (DTT; Sigma–Aldrich), alkylated with 100 mM iodoacetamide (Thermo Scientific), and separated on SDS-PAGE. Each lane was cut into six pieces and digested with trypsin (Promega). Sucrose gradient PARP1 immunoprecipitation samples were eluted from beads with 8 M urea in 50 mM Tris (pH 8.0), reduced in 20 mM DTT, alkylated with 40 mM iodoacetamide, quenched with 10 mM DTT, diluted to less than 1 M urea with 50 mM Tris (pH 8.0), and digested with trypsin (Promega). Samples were run on a Thermo Fisher Q Exactive coupled with Exilgent LC system (AB Sciex) over a 1 hr gradient. Peptide identification and quantification was performed with MaxQuant v1.3.05 (Cox et al., 2009).
**KAT5 In Vitro Activity Assay**

Beads from KAT5 immunoprecipitations were mixed with 0.2 mg/ml histone H4 peptide (Signalchem) and 10 μM acetyl-coenzyme A (EMD Millipore), incubated for 30 min at 30 °C, and spotted onto nitrocellulose. The membrane was blocked and probed with anti-acetyl H4(K8) (Cell Signaling Technology). A far-red fluorescent secondary antibody was used for imaging on the Odyssey (LI-COR Biosciences). Background subtraction and quantification was done with the ImageJ MJB plugin suite (http://rnb.info.nih.gov/i/plugins/mjbf/index.html).

**RNAi**

Cells were transfected with siRNA using either Lipofectamine 2000 or RNAiMax (Invitrogen) according to the manufacturer’s protocol. RNAi was done with a double transfection protocol over 7 days total. Cells were allowed to recover for 3 days after the first transfection, retreated, and then allowed to grow for an additional 3 days. All siRNAs were ordered from Thermoflag Scientific, and the following siRNAs were used: PARP1 ON-TARGETplus (GAUUUCUCUGGUGUGUAGU or CCAUUAGGGUAAUCCUGU), EP400 siG ENOME siRNA SMARTpool, KAT5 siGENOME siRNA SMARTpool, RUVBL1 siGENOME siRNA SMARTpool, and DMAP1 siGENOME siRNA SMARTpool.

RNA efficiency was tested via RT-PCR or immunoblot. Total RNA was extracted from cells with the QIAGEN RNeasy kit, and RT-PCR was done with the One-step RT-PCR kit (QIAGEN) with the following primer pairs: PARP1, GCCCTAAGGCTCAGAAGCA and AGGTTCCCCGAGGATCGAGGAT; EP400, TCCACGACGCAACCATTCC and ATTCCTCCTGTGGGCTGCG; KAT5, AGGGAGAATACTGAGGGG and CACCTCTCGTTGGTGACG; RUVBL1, TG GTTTACGCCAAGCTGTTTT and CTCTTTATTCCGACGCAAAATG; DMAP1, CAGGCCCCTCTAAAGGAAGC and GCTTCTCAGAATGGGCGGCTG; and PARP2, AGGTCATGGGGCCGCAAAGG and ACGAGTCCAAGGTCACTGG.

**Transcriptional Profiling**

Cells were treated with DMSO, olaparib, or ABT-888 (Selleck Chemicals) for 6 or 48 hr and harvested via trypsinization. Total RNA was extracted using the QIAGEN RNeasy Plus kit. Samples were submitted to the Boston Children’s Hospital Microarray Core Facility for transcriptional profiling using Illumina HT-12 beadchips. Initial data analysis including normalization and background subtraction was performed with the Illumina GenomeStudio software package. The quantile-background subtracted data were used for further analysis using the R platform.

**ACCESSION NUMBERS**

Transcript-profiling data have been deposited to the NCBI Gene Expression Omnibus and are accessible under accession numbers GSE58844 and GSE56400.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.009.

**AUTHOR CONTRIBUTIONS**

K.A.K., J.A.S., and T.J.M. designed the study. K.A.K. performed the experiments and analyzed the data. R.J. analyzed the transcriptional data. K.A.K. and T.J.M. wrote the manuscript.

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