BAP1 Has a Survival Role in Cutaneous Melanoma

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Although the pattern of *BAP1* inactivation in ocular melanoma specimens and in the BAP1 cutaneous melanoma (CM)/ocular melanoma predisposition syndrome suggests a tumor suppressor function, the specific role of this gene in the pathogenesis of CM is not fully understood. We thus set out to characterize BAP1 in CM and discovered an unexpected pro-survival effect of this protein. Tissue and cell lines analysis showed that *BAP1* expression was maintained, rather than lost, in primary melanomas compared with nevi and normal skin. Genetic depletion of *BAP1* in melanoma cells reduced proliferation and colony-forming capability, induced apoptosis, and inhibited melanoma tumor growth *in vivo*. On the molecular level, suppression of BAP1 led to a concomitant drop in the protein levels of survivin, a member of anti-apoptotic proteins and a known mediator of melanoma survival. Restoration of survivin in melanoma cells partially rescued the growth-retarding effects of BAP1 loss. In contrast to melanoma cells, stable overexpression of BAP1 into immortalized but non-transformed melanocytes did suppress proliferation and reduce survivin. Taken together, these studies demonstrate that BAP1 may have a growth-sustaining role in melanoma cells, but that its impact on ubiquitination underpins a complex physiology, which is context and cell dependent.

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INTRODUCTION

The BRCA1-associated protein 1 (*BAP1*) gene is a recent addition to the canon of high-risk melanoma susceptibility genes. Many groups have described germline *BAP1* alterations in families predisposed to cutaneous melanoma (CM) and ocular melanoma among other malignancies (Harbour *et al.*, 2010; Abdel-Rahman *et al.*, 2011; Wiesner *et al.*, 2011; Njauw *et al.*, 2012). To date, both heritable and acquired mutations in *BAP1* have been deleterious with loss-of-heterozygosity described in melanoma tumor specimens (Wiesner *et al.*, 2011; Njauw *et al.*, 2012). This genetic pattern suggests a tumor suppressor function for the BAP1 protein. However, unlike ocular melanomas, CMs do not commonly harbor *BAP1* mutations outside of the familial context (Harbour *et al.*, 2010; Wiesner *et al.*, 2011; Njauw *et al.*, 2012). More importantly, the role of BAP1 in the pathogenesis of sporadic CM has yet to be fully characterized.

Functional analyses of BAP1 have yielded conflicting results. Early experiments found that BAP1 enhanced BRCA1-mediated inhibition of breast cancer cell growth (Jensen *et al.*, 1998). BAP1 has also been reported to be inactivated in about 15% of renal cell carcinomas and has been shown to be growth suppressive in functional assays (Pena-Llopis *et al.*, 2012). With the recognition that BAP1 directly interacts with HCF-1, there were hints that BAP1 could also have a positive proliferative role (Machida *et al.*, 2009). These findings were further substantiated by genome-wide RNA suppression screens, which established BAP1 as an essential proliferation and cell cycle gene (Kittler *et al.*, 2007; Schlabach *et al.*, 2008). Even among uveal melanomas, where the prevalence of deleterious mutations remains the highest, recent studies suggest that BAP1 is not functionally suppressive and that the biology of this deubiquitinase is highly complex (Matatall *et al.*, 2013).

We thus set out to better understand the role of BAP1 in CM—one of the signature cancers in the BAP1 tumor predisposition syndrome. Using a combination of genetic and functional studies, we provide evidence that BAP1 may be an important growth-sustaining protein that is linked to the regulation of survivin—a known anti-apoptotic factor in melanoma (McKenzie and Grossman, 2012).

RESULTS

Basal expression level of *BAP1* in melanomas

We first examined the messenger RNA (mRNA) levels of *BAP1* in primary melanocytic tumors using a set of 223 melanomas, 11 nevi, and 6 samples of normal tissue (Harbst *et al.*, 2012) and found no significant difference in relative *BAP1* expression (Figure 1a), although levels may be slightly lower in mucosal melanomas. When stratified by tumor features (Figure 1b), *BAP1* expression was increased in thin melanomas (<1 mm) and decreased in thick melanomas (>4 mm) compared with nevi and normal tissue; however, the

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Abbreviations: mRNA, messenger RNA; NTC, nontargeting control; OM, ocular melanoma; PHF, primary human fibroblast; PHM, primary human melanocyte

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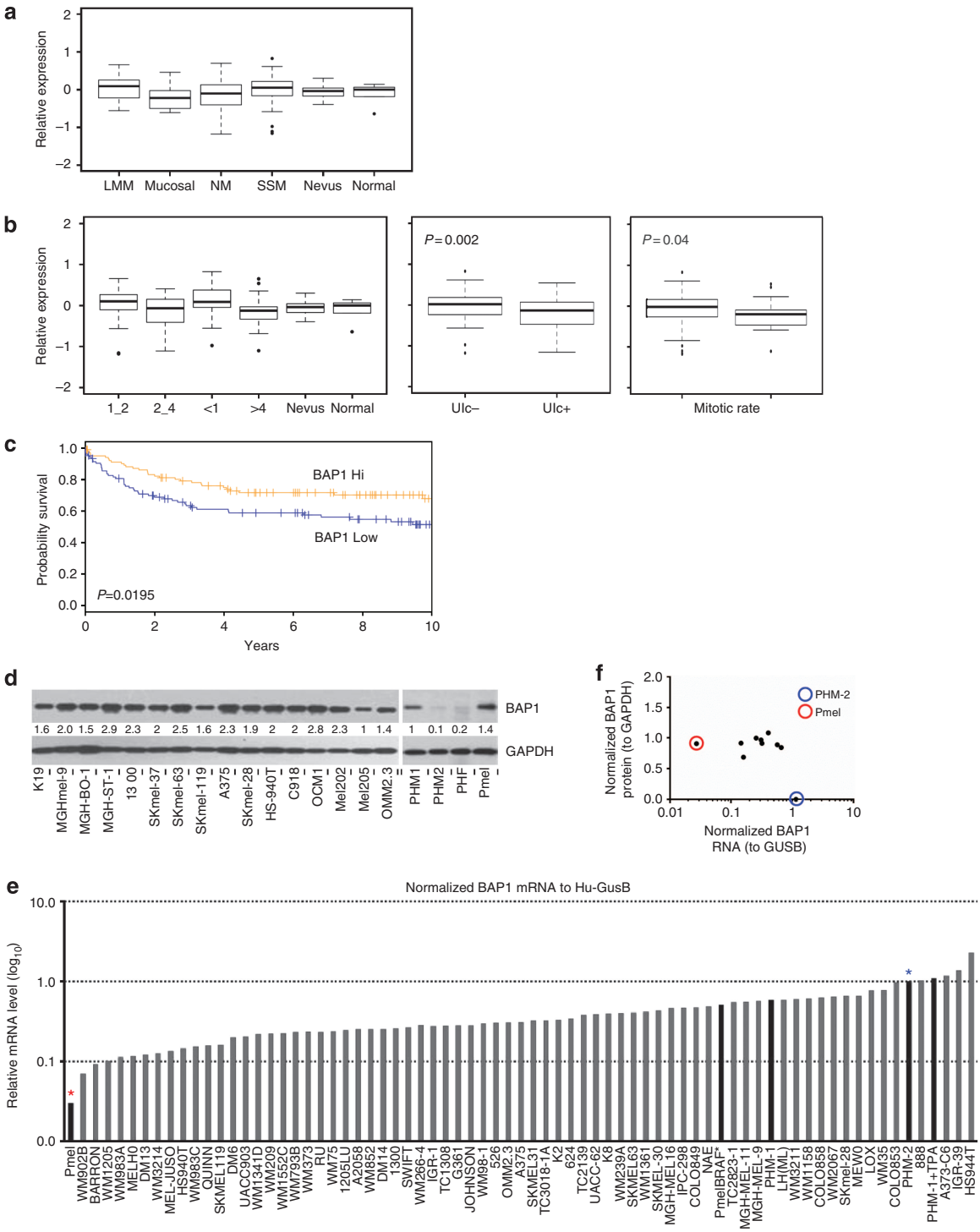

Figure 1. BAP1 expression in primary cutaneous melanomas and melanoma lines. (**a**) BRCA1-associated protein 1 (*BAP1*) expression in histological types of melanoma. (**b**) *BAP1* expression melanomas stratified by Breslow thickness, *BAP1* expression in relation to ulceration, and the mitotic rate. (**c**) Survival differences between *BAP1* high (>mean gene expression across the melanomas) and *BAP1* low tumors (⩽mean gene expression across the melanomas). (**d**) Western blot analysis showing relative protein levels of BAP1 in a collection of 16 melanoma cell lines, primary human melanocytes (PHMs), primary human fibroblasts (PHF), and an immortalized non-transformed human melanocytes (Pmel). (**e**) Ranked *BAP1* RNA levels (normalized to human *GUSB*) in a panel of melanoma lines and PHM-1, PHM-2, and Pmel. (**f**) Correlation between normalized BAP1 protein (to GAPDH) and normalized *BAP1* RNA (to Hu-*GUSB*). Pmel (red circle and red asterisk) shows low RNA levels but strong protein expression, whereas PHM-2 (blue circle and blue asterisk) shows high RNA levels with a near absence of detectable protein. LMM, lentigo maligna melanoma; mRNA, messenger RNA; NM, nodular melanoma; SSM, superficial spreading melanoma.

difference across all groups was nonsignificant. Lower *BAP1* expression was observed in primary melanomas with ulceration ($P=0.002$) and higher mitotic rate ($\geqslant 6$ mitoses versus <6 mitoses; $P=0.04$). Finally, when supervised by outcome, primary melanomas with low *BAP1* expression exhibited a worse prognosis compared with those with high *BAP1* levels (Figure 1c), although these results were likely confounded by its association with ulceration and the high mitotic rate. We then examined *BAP1* levels in an independent set of metastatic melanomas (Gene Expression Omnibus GDS1375) and identified a significant increase in the levels of *BAP1* among metastatic cases compared with normal tissue and nevi (Supplementary Figure S1a online).

The levels of BAP1 in proliferating melanoma cells were then determined. We first assessed BAP1 protein levels in 16 melanoma lines, 2 independent primary human melanocyte lines (PHM-1 and PHM-2), a primary human fibroblast (PHF) line, and an immortalized, but non-transformed, melanocyte line (Pmel; Figure 1d). Except for PHM-2 and the PHF, there was a robust protein expression in all samples. RNA expression was then examined in a broader panel of lines by quantitative PCR (Figure 1e) and was found to be well sustained in all the melanoma lines. Interestingly, protein levels were generally constant, despite a gradient of RNA expression (Figure 1e). One of the primary melanocyte lines, PHM-2, showed negligible protein expression (Figure 1f) even with relatively high RNA content (blue asterisk, blue circle), whereas the immortalized melanocyte line, Pmel, demonstrated strong BAP1 protein levels despite low RNA expression (red asterisk, red circle). These findings indicate that melanoma cells, but not necessarily primary cells, preserve the amount of intracellular BAP1. To replicate these findings and to put melanoma in the context of other proliferating cancer cells, we used the Cancer Cell Line Encyclopedia ($N=1{,}036$ cancer lines) and found that the median expression of *BAP1* in 61 melanoma lines ranked ninth among the 37 cancer cell types (Supplementary Figure S1b online) and was significantly higher compared with all non-melanoma lines (Supplementary Figure S1b online; 7.59 vs. 7.33; $P<0.001$, Student's *t*-test). Thus, BAP1 appears central to the survival of melanoma cells, although its role in primary cells is less clear.

BAP1 depletion abrogates melanoma growth

To test the hypothesis that BAP1 contributes to the melanoma cell maintenance, we examined the effects of BAP1 depletion on the growth kinetics of melanoma cells. As shown in Figure 2, depletion of BAP1 in two BRAF(V600E)-mutant lines (A375 and SKmel-28, Figure 2a) and two NRAS(Q61R)-mutant lines (SKmel-119 and SKmel-63, Figure 2b) led to marked reductions in melanoma proliferation. These were also accompanied by significant decreases in the colony-forming capacity of the BAP1-depleted cells (Figure 2c). Finally, we investigated the effects of BAP1 loss on tumor growth *in vivo* using two sh(BAP1)-suppressed lines (A375 and C918). As shown in Figure 2d, BAP1 depletion diminished the tumorigenicity of melanoma xenografts in immunocompromised mice. Examination of the tumor specimens demonstrated less Ki67 and more TUNEL staining in the two sh(BAP1) tumors compared with the control tumors (Supplementary Figure S2 online). This suggests that BAP1 loss can produce similar anti-proliferative and pro-apoptotic effects *in vivo* as found *in vitro*.

For the cell cycle and apoptosis assays (Figure 3), A375 [BRAF(V600E)], SKmel-119 [NRAS(Q61R)], and C918 (uveal melanoma) cells were used. In 10% serum (Figure 3a), suppression of BAP1 led to G_2/M arrest with modest G_1/S effects. However, in 2.5% serum (Figure 3b), there were appreciable increases in apoptosis as measured by subG_1 fractionation; this apoptotic response was independently confirmed using FITC-Annexin staining (Figure 3c). These results indicate that both cell cycle arrest and apoptosis may contribute, in part, to the proliferative shut down observed with BAP1 suppression *in vitro*.

BAP1 regulates survivin

We set out to identify potential mediators of BAP1-dependent survival. As one of the IAP family members—*BIRC5* or survivin—has been implicated both as a viability factor in melanoma and a target of BAP1 regulation in U2OS cells (Yu *et al.*, 2010), we hypothesized that BAP1 depletion may have an effect on survivin levels. As shown in Figure 4a, *BAP1* suppression led to a marked loss of survivin protein levels in three of four cell lines examined (A375, SKmel-28, and C918); quantitative PCR confirmed a concomitant loss of *BIRC5* mRNA in A375 and SKmel-28, but not in C918, melanoma cells. In one cell line (SKmel-119), there was a rise in *BIRC5* mRNA and survivin protein levels. Thus, BAP1 effects are cell context dependent and may exist at both transcriptional and post-transcriptional levels. To determine whether the loss of survivin was a necessary component of the growth inhibition observed with BAP1 depletion, we overexpressed survivin in the context of BAP1 loss. As shown in Figure 4b, there was a consistent rescue of cell growth in the A375(shBAP1) cells when survivin was overexpressed (Figure 4b; A375 (shBAP1/BIRC5-OE) versus A375 (shBAP1)). In addition, if *BIRC5* is under BAP1 regulation, then one might expect a direct relationship between the levels of the two genes. As shown in Supplementary Figure S3a online, there was indeed a significant correlation between *BIRC5* and *BAP1* mRNA levels in the CCLE melanoma data set ($P=0.0026$; $N=62$). Moreover, *BIRC5* loss was similarly associated with a worsened outcome in the 223 primary melanomas (Supplementary Figure S3b online; $P=0.0009$). Taken together, these findings suggest that BAP1 loss appears to have a negative impact on survival factors and that downregulation of species such as survivin may be one component of the BAP1 effect.

To examine more specific role of ubiquitination, we performed a proteasome/protein clamp experiment using A375 and C918 cell lines, both of which showed a loss of survivin protein levels with BAP1 depletion and yet contrasting effects on the *BIRC5* mRNA level. Briefly, control and BAP1-depleted A375 and C918 cells (i.e., A375 (nontargeting control (NTC))/A375 (shBAP1) and C918 (NTC)/C918 (shBAP1)) were subjected to 6-hour pre-treatment with proteasome inhibitor MG132 (25 μM) to enrich for ubiquitinated proteins. As shown in Supplementary Figure S4 online, total

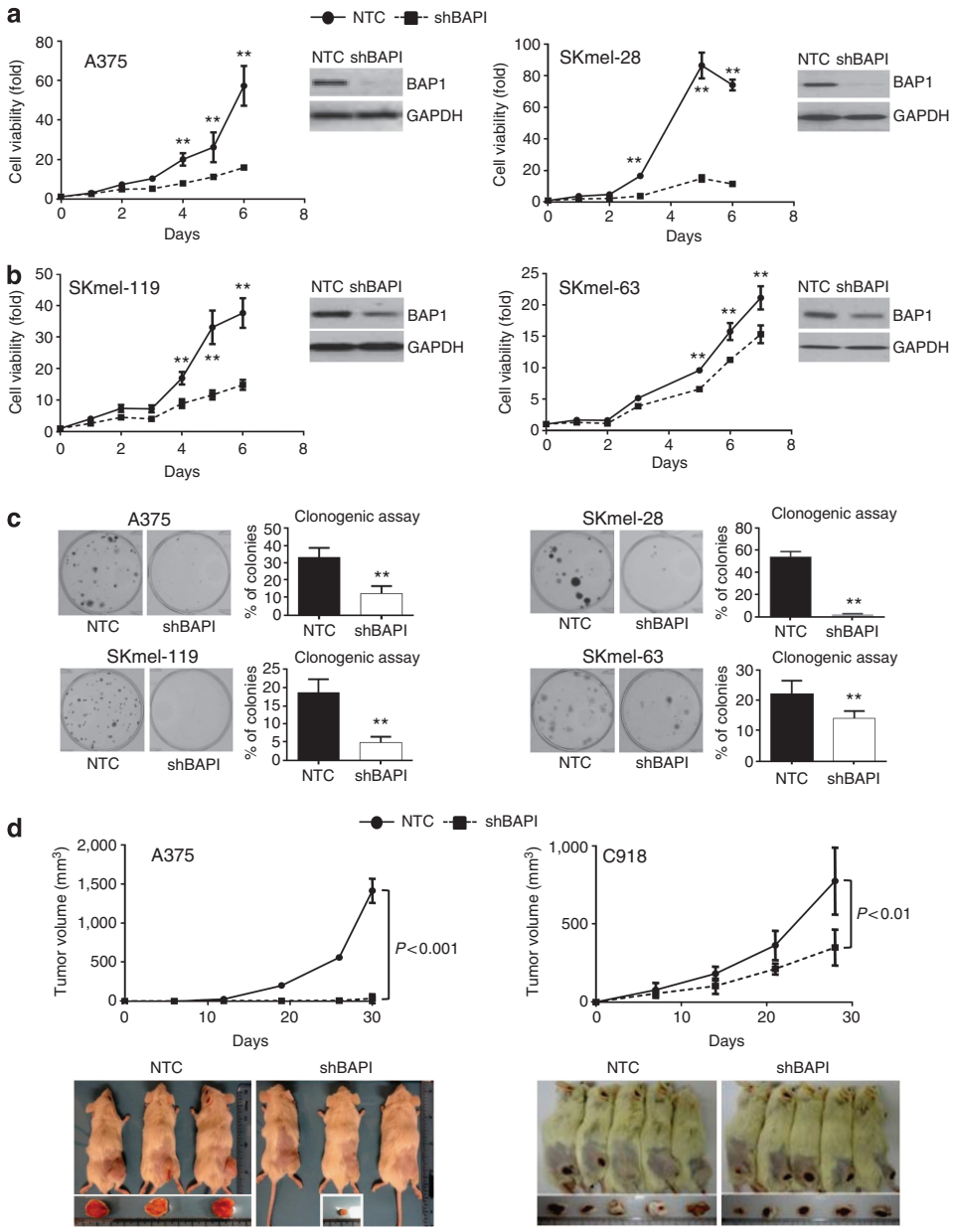

Figure 2. BAP1 depletion leads to melanoma growth suppression. The short-hairpin RNA (shRNA)-mediated suppression of BRCA1-associated protein 1 (BAP1) results in reduced *in vitro* proliferation in four cutaneous melanoma cell lines. (**a**) A375 and SKmel-28 harbor BRAF (V600E) mutations, whereas (**b**) SKmel-119 and SKmel-63 contain NRAS (Q61R) and NRAS (Q61K) mutations, respectively. Error bars represent standard error of mean from triplicate samples. (**c**) Loss of BAP1 is also associated with diminished colony-forming capability. Error bars represent ± standard deviation (SD), from at least three independent experiments. *$P<0.05$ and **$P<0.01$ by Student's *t*-test. (**d**) Effects of BAP1 depletion on tumor growth in a xenograft model. One million A375(NTC), A375(shBAP1), C918(NTC), and C918(shBAP1) cells were implanted subcutaneously in NOD-SCID-IL2G-null mice with matrigel in a 1:1 ratio and observed over the indicated time period. With A375 (cutaneous melanoma), three animals were used in each arm, whereas for C918 (uveal melanoma) five animals were used in each arm. Error bars represent ± SD, in tumor volume. *$P<0.05$ and **$P<0.01$ by Student's *t*-test. NTC, nontargeting control.

protein ubiquitination was markedly increased by the MG132 treatment. The cells were then removed from MG132 exposure and treated with the protein synthesis inhibitor, cycloheximide (25 μg ml^{-1}; transfer time = 0). The levels and decay of BAP1 regulated protein(s) were then analyzed at 1, 2, 4, and 6 hours after cycloheximide exposure using Western blotting (Figure 4d). In untreated A375 and C918 cells, the loss of BAP1 was associated with a marked reduction in survivin levels (Figure 4d, control ''C'' lanes), consistent with the prior analysis. MG132 treatment alone (''+M'' versus ''C'') led to increased survivin, suggesting that proteasomal degradation modulates survivin levels. Both A375(shBAP1) and C918(shBAP1) lines exhibited appropriate survivin decay upon release of MG132 and inhibition of new protein synthesis by cycloheximide, despite having attenuated survivin protein levels at baseline. As BAP1 depletion led to

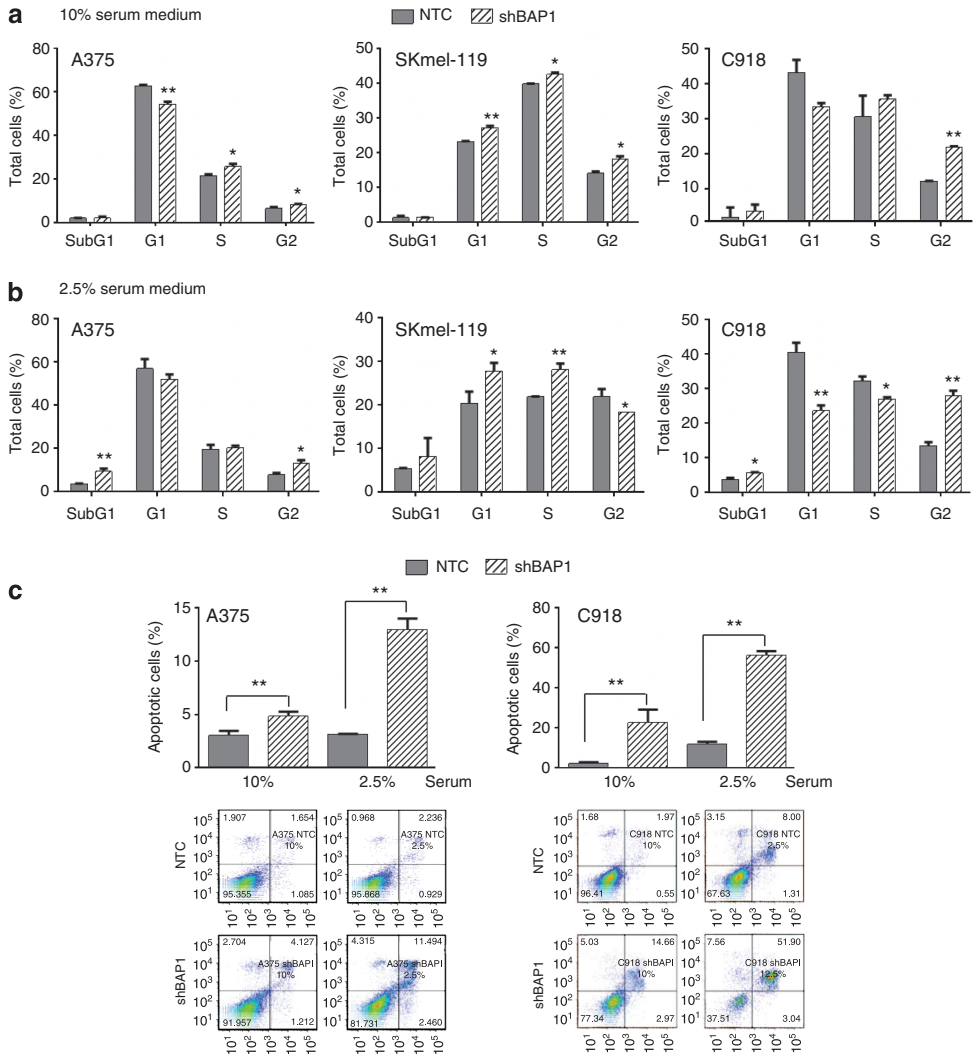

Figure 3. BAP1 depletion causes cell cycle arrest and apoptosis. Analysis of cell cycle progression and apoptosis was performed on the 6th or 7th day following short-hairpin RNA (shRNA)-mediated BRCA1-associated protein 1 (BAP1) silencing. Cell cycle analysis in (**a**) 10% and (**b**) 2.5% serum medium using control cells (gray shading) and shBAP1 knockdown cells (hatched shading). Error bars represent standard error of mean (SEM) from triplicate samples and three experimental replicates are shown. A375 and SKmel-119 are cutaneous melanomas, and C918 is an ocular melanoma used for comparison; $*P<0.05$ and $**P<0.01$ by Student's *t*-test. (**c**) FITC-Annexin staining of cultured control and BAP1-depleted cells in both 10% and 2.5% serum. DNA fragmentation may underestimate the level of apoptosis, especially if the total DNA content is elevated from G_2/M arrest. Error bars represent SEM from triplicate samples both replicates shown; $**P<0.01$ by Student's *t*-test. NTC, nontargeting control.

diminished resting levels of survivin but did not abrogate survivin decay upon MG132/cycloheximide treatment, BAP1 likely participates in the homeostatic maintenance of survivin levels through other mechanisms beyond simple deubiquitination. Furthermore, co-immunoprecipitation experiments were carried out but did not demonstrate direct protein–protein interaction between BAP1 and survivin (data not shown).

BAP1 suppresses non-transformed melanocytes

Our findings so far support a role for BAP1 in melanoma cell viability. However, BAP1's effects may be different in non-transformed cells. We thus chose to restore BAP1 in cells that appear to exist in relative BAP1 deficiency. Studies were first initiated in PHM-2, which lacked BAP1 protein, but neither control vector nor BAP1-expressing cells could be sufficiently recovered for experimentation. We next turned to an immortalized but non-transformed melanocyte line, Pmel, which had lower RNA levels of BAP1 compared with any of the melanoma cells. As shown in Figure 5a, stable introduction of BAP1 into Pmel cells (i.e., Pmel(BAP1)) reduced proliferation, colony-forming capacity, and survivin levels. Interestingly, Pmel (shBAP1) cells also exhibited a drop in survivin levels, *albeit* less significant than in Pmel(BAP1), and a similar growth-suppressed phenotype (Figure 5a). On the other hand, ectopic expression of BAP1 in three distinct melanoma cell lines led to a modest increase in survivin and proliferation (Figure 5b).

DISCUSSION

Although inactivating germline mutations of *BAP1* have been described in families prone to CM and ocular melanoma

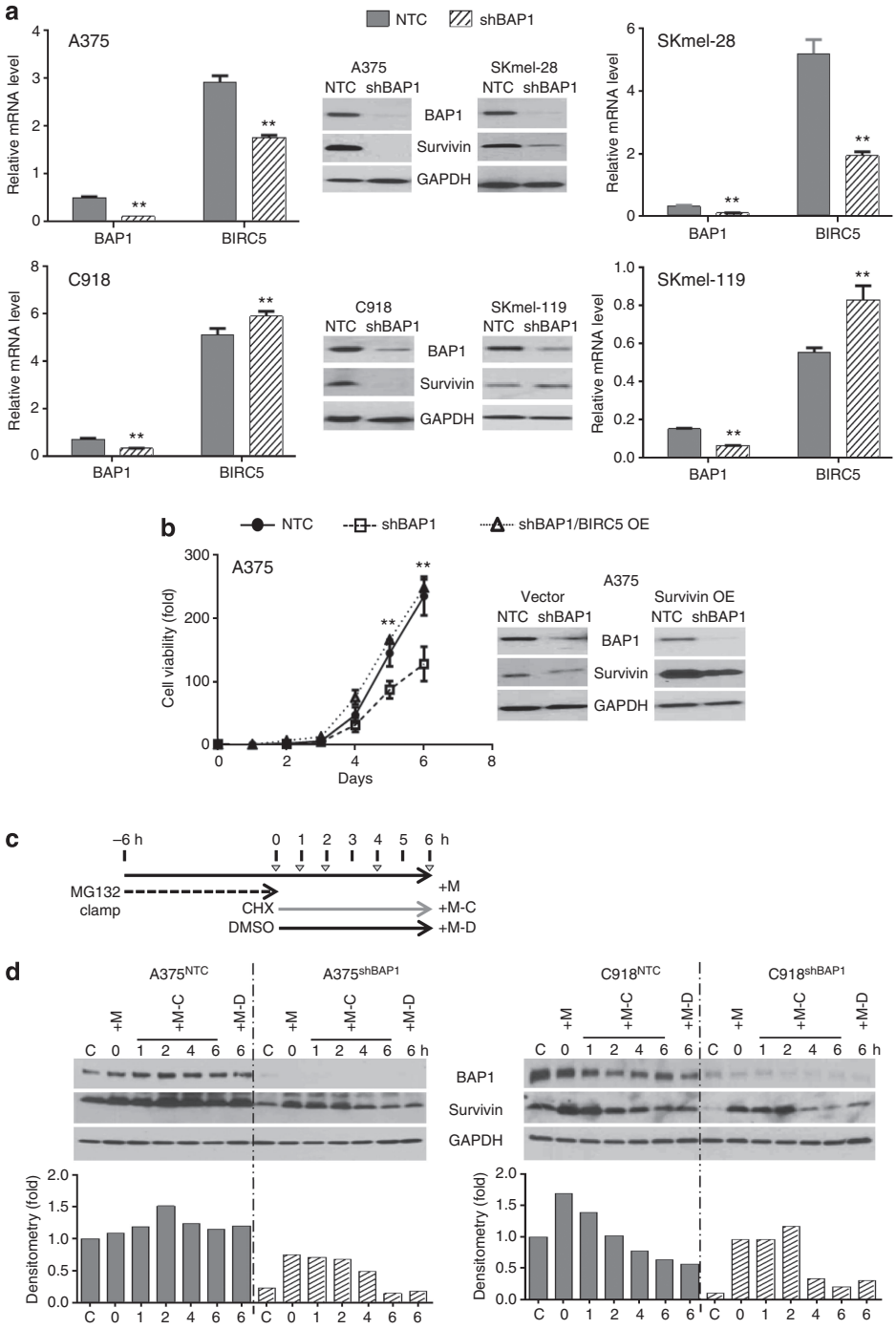

Figure 4. BAP1 suppression is associated with survivin depletion. (**a**) RNA and protein levels of *BIRC5* (survivin) upon BRCA1-associated protein 1 (BAP1) suppression in four melanoma lines. There is evidence of near total survivin loss at the protein level in A375, SKmel-28, and C918 but not SKmel-119. Error bars represent standard error of mean from triplicate samples; $*P<0.05$ and $**P<0.01$ by Student's *t*-test. (**b**) Survivin overexpression (OE) in A375 cells led to the rescue of the *in vitro* growth arrest induced by shBAP1. (**c**) Schematic diagram of experimental design for the ubiquitination assay. Indicated cell lines were incubated with MG132 (25 μM) for 6 hour, followed by the removal of MG132 and the addition of cycloheximide (CHX, 25 $\mu g\,ml^{-1}$) for the designated time intervals. "C" represents control cells that were exposed to neither MG132 nor CHX. "+M" indicates cells that were exposed to only MG132 and not CHX; for these cells, lysate was collected at time = 0. "+M-C" represents cells that were exposed to MG132 and then switched to CHX; lysates were collected at 1, 2, 4, and 6 hours post CHX switch. "+M-D" represents cells that were exposed to MG132 and then DMSO control for 6 hour. (**d**) The effect of BAP1 depletion on survivin and GAPDH protein levels as measured by Western blotting. If BAP1 directly deubiquitinated survivin, then survivin decay should be accelerated with BAP1 depletion. Even though the absolute levels of BAP1 appear to be lower in the shBAP1 lines, survivin degradation appears similar. Error bars represent standard error of mean of triplicate samples. The experiments were performed three times with similar results; $**P<0.01$ by Student's *t*-test. mRNA, messenger RNA; NTC, nontargeting control.

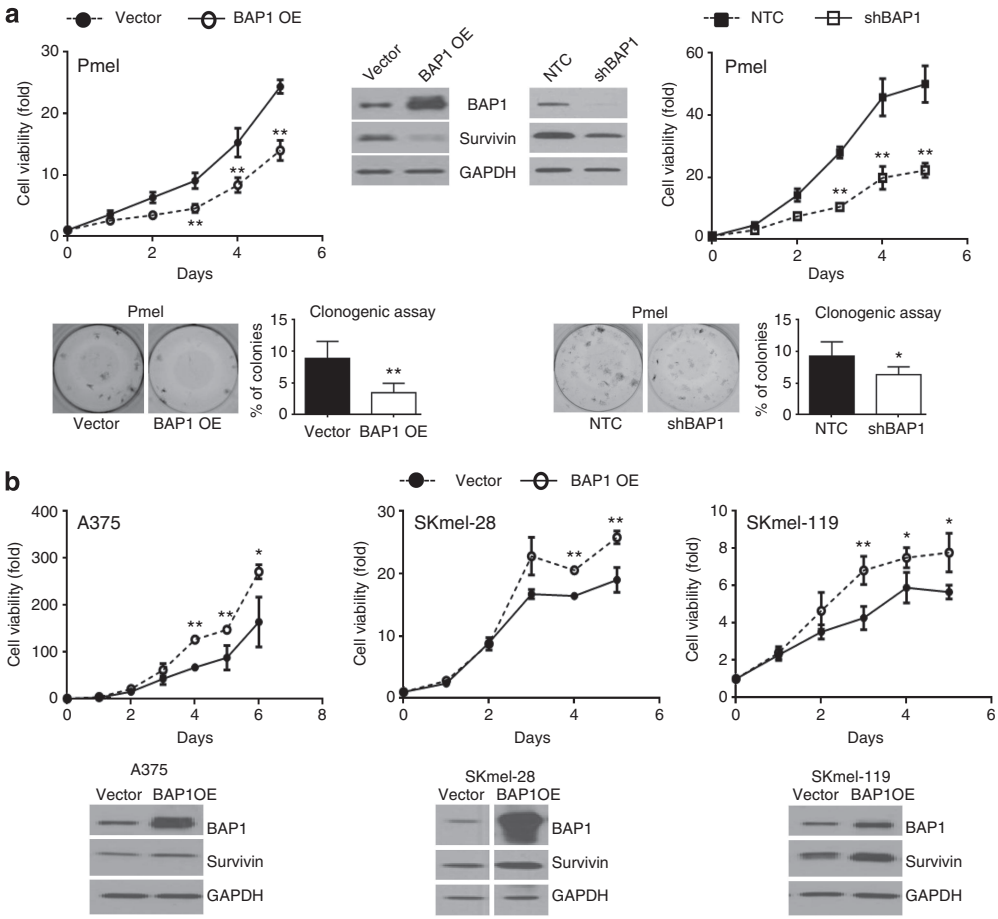

Figure 5. BAP1 overexpression in Pmel and melanomas. (**a**) Effects of BRCA1-associated protein 1 (BAP1) overexpression (BAP1 OE) and depletion (shBAP1) on Pmel proliferation, survivin levels, and colony-forming capacity. Both BAP1 elevation and suppression in Pmel cells were associated with a decrease in colony formation, proliferation, and levels of survivin. (**b**) Overexpression of BAP1 in three melanoma lines had more stimulatory effects on proliferation and survivin. The experiments were performed three times with similar results; $*P<0.05$ and $**P<0.01$ by Student's *t*-test. NTC, nontargeting control.

(Abdel-Rahman *et al.*, 2011; Wiesner *et al.*, 2011; Njauw *et al.*, 2012), the role of *BAP1* in the pathogenesis of CM outside of the familial context is not fully known. Somatic mutations of *BAP1* occur in only 5% of sporadic primary melanomas (Wiesner *et al.*, 2011), suggesting that sustained BAP1 activity may have a critical function in tumor maintenance. In this study, we discovered that melanoma cells appear to defend their BAP1 protein levels, despite a wide range of *BAP1* RNA content. Furthermore, BAP1 appears to have a role in regulating the levels of survival genes, such as *BIRC5*/survivin, and in maintaining the growth of at least a subset of melanomas.

BAP1 mRNA levels do not appear to be significantly diminished in primary melanomas compared with nevi and normal skin, although lower expression levels do correlate with adverse features, such as the mitotic rate and ulceration, and a worse outcome. Among metastatic cases, *BAP1* mRNA levels appear to be higher compared with those found in nevi and normal skin. Using immunohistochemistry, Murali *et al.*, (2013) also recently reported that BAP1 loss occurred in only 5% of primary melanomas and was associated with worsened survival—a finding that resonates with our results. Interestingly, reduced BAP1 was observed more often in the desmoplastic variant of melanomas where there is greater fibroblastic investment. Although preliminary, PHFs in our survey (Figure 1) expressed no BAP1 and may therefore account for the lower observed staining. Overall, *BAP1* is rarely mutated in primary CM and its expression is generally sustained, which is in sharp contrast to primary uveal melanomas where over half of the cases exhibit a total absence of BAP1 (Shah *et al.*, 2013). Thus, the biologic function of BAP1 may be different between the two types of melanoma cells.

A second, somewhat unexpected finding is that BAP1 appears to be a survival factor in melanoma cells. BAP1 depletion diminished proliferation and enhanced apoptosis, both *in vitro* and *in vivo*, and phenotypically inhibited tumor growth in mice xenografts. These functional studies may explain the consistent high level expression in nearly all melanoma lines. Others have also observed growth arrest in non-melanoma cancer cells (Dalinghaus *et al.*, 1991; Kittler *et al.*, 2007; Machida *et al.*, 2009, Testa *et al.*, 2011) with loss of BAP1. However, BAP1 is not universally required for cellular viability as some PHMs and PHFs do not appear to

express appreciable levels of BAP1 protein. Paradoxically, recent findings have confirmed that BAP1 also promotes growth and differentiation even in uveal melanomas where loss of BAP1 expression is a common event (Matatall *et al.*, 2013). Germline *Bap1* deletion in mice is lethal during embryogenesis, indicating that fetal growth and development requires this gene (Dey *et al.*, 2012). Thus, the specific phenotype that results from BAP1 disruption may be dictated by the balance of growth-restricting and growth-promoting effects that are regulated by BAP1. This context-specific effect is supported by our early results with the Pmel cells, suggesting that BAP1 may harbor altogether different properties in non-transformed cells.

Our studies reveal that BAP1 also impinges on the intracellular metabolism of survivin, although the precise mechanistic details are still under investigation. In a microarray profiling experiment, survivin mRNA was also reported to be significantly decreased in U2OS cells that have been depleted of BAP1 (Yu *et al.*, 2010). Gene expression studies, however, may not fully uncover all BAP1 targets. In our hands, C918 uveal melanoma cells show sustained, if not slightly higher, *BIRC5* mRNA levels upon BAP1 suppression, despite a near absence of BAP1 protein levels (Figure 4a). Thus, it appears that BAP1 could regulate target proteins at both transcriptional and the post-transcriptional levels (Yu *et al.*, 2010; Bott *et al.*, 2011; Carbone *et al.*, 2013).

There are several limitations to our study. As BAP1 is differentially expressed in primary melanocytes, the role of BAP1 in non-malignant cells may be functionally distinct from that in their malignant counterparts. This could resolve the apparent paradox between germline predisposition and cellular dependence. Studies are underway to examine the cooperative effects of BAP1 loss, or gain, with other oncogenic alleles in human melanocytes. In addition, the type and position of the reported mutations may bear on the suppressive or oncogenic nature of the altered BAP1 proteins. We are in the process of performing a more refined genotype–phenotype correlation on the cellular level. Finally, the biology of BAP1 will likely require a full annotation of BAP1 targets, both as direct enzymatic substrates and as indirect co-regulators of transcription.

In summary, we provide early but provocative evidence that BAP1 has an important growth-sustaining role in many CMs and that some of the growth-retarding effects of BAP1 loss may be mediated by viability factors such as survivin. These studies also suggest that BAP1 could have a different role in non-malignant cells and highlight the complex nature of genetic and functional attributes of BAP1 biology.

MATERIALS AND METHODS

Cellular proliferation, colony formation, cell cycle, and apoptosis assays

These assays were performed as previously described by our laboratory (Udayakumar *et al.*, 2011; Ji *et al.*, 2012; Ji *et al.*, 2013). The PrestoBlue cell viability assay was performed as per the manufacturer's instruction (Life Technologies, Grand Island, NY). After trypsinization and trypan Blue staining (Sigma-Aldrich, St Louis, MO), viable cells were plated at a density of 10^3 cells per well in black 96-well plates. Cell proliferation assays were performed at 24-hour intervals for up to 6 days. Briefly, PrestoBlue dye was added, at 1/10 of the culture medium volume, and incubated for 10 minutes at 37 °C. The reaction was stopped with 15 µl per well of 1 × SDS (Life Technologies), and the fluorescence was measured at 540 nm excitation and 590 nm emission using a microplate reader (SpectraMaxplus 1311, Molecular Devices, Sunnyvale, CA). Raw fluorescence values were subtracted from the background of the no-cell control wells for each experimental well. All experiments were performed at least three times in triplicate under each condition.

For the colony formation assay, short-hairpin RNA-BAP1 and non-target shRNA control (NTC)-transduced melanoma cells were plated at 100–200 cells per ml per well into 12-well plates and kept in a humidified CO_2 incubator at 37 °C for 15–20 days (Udayakumar *et al.*, 2011; Ji *et al.*, 2012; Ji *et al.*, 2013). Cells were then washed with cold 1 × phosphate-buffered saline and fixed in 100% methanol for 30 minutes at room temperature and stained with crystal violet 0.5% w/v for 30 minutes. The stained colonies were counted under the stereo microscope and compared with a non-targeted control. Colony-forming capacity or plating efficiency was expressed as a ratio of the number of colonies consisting of $\geqslant$50 cells to the number of cells seeded.

Cells were processed for cell cycle and apoptosis experiments concurrently. Cell cycle analyses were performed to evaluate the distribution of cells in various cell cycle phases (subG$_1$, G$_1$, S, and G$_2$/M) by measuring the DNA content of nuclei labeled with propidium iodide (Life Technologies). Briefly, BAP1-depleted and control A375, SKmel-119, and C918 viable cells were plated at 0.3×10^6 cells per well in six-well tissue culture plates and incubated for 6–7 days at 37 °C in 5% CO_2. At 6–7 days post lentiviral infection, the cells were trypsinized then fixed with ice-cold ethanol final 70% (v/v) at −20 °C overnight. Cells were centrifuged and washed twice with cold 1 × phosphate-buffered saline and re-suspended in 0.5 ml of solution (20 µg ml^{-1} of propidium iodide and 200 µg ml^{-1} of RNase in 0.1%Triton X-100) in phosphate-buffered saline (RNAse, Becton Dickinson, San Jose, CA) and incubated at room temperature in the dark for 30 minutes. Samples were then subjected to FACS (BD FACS Calibur flow cytometer, BD Biosciences, Sparks Glencoe, MD). Using the FlowJo, version 7.6.5 software (Ashland, OR) the Watson model was used to calculate the percentages of cells in various cell cycle phases. All experiments were performed at least three times in triplicate.

For apoptosis, subG$_1$ fractionation of cells with reduced DNA content was determined at the time of cell cycle analysis. In addition, apoptosis of short-hairpin RNA-BAP1 and NTC-transduced melanoma cells (grown in complete (10%) or reduced (2.5%) serum media) were determined using the Alexa Fluor 488 annexin-V conjugate detection kit as per the manufacturer's instructions (Life Technologies). Briefly, melanoma cells (A375 and C918) were plated (0.3×10^6 cells per well per ml) into six-well plates and incubated in a 5% CO_2 incubator at 37 °C for 6–7 days after transduction. Cells were then trypsinized, centrifuged at 300*g* for 5 minutes, and stained with Alexa Fluor 488 annexin-V and propidium iodide for 15 minutes at room temperature in the dark. A total of 10,000 events were analyzed for each sample on a flow cytometer FACS Verse, and results were analyzed using FlowJo, version 7.6.5 software. The cell cycle and apoptosis assays were performed in parallel and in triplicate for each condition.

Xenograft tumor growth assay

Nod-SCID-gamma mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously with NTC-transduced or short-hairpin RNA-BAP1-transduced A375 or C918 cells. The cells were mixed with matrigel in 1:1 ratio (1×10^6 cells per mice in three to five mice per group). Animal body weights and tumor development were monitored and dimensions were measured by a Mitutoyo caliper (MSC, Melville, NY) once to twice per week. Tumor volume was calculated using $mm^3 = \text{length} \times \text{width}^2 \times 0.5$. Animals were maintained in well-ventilated animal facility and tested in accordance with the MGH Animal Care and Use Committee guidelines. Data were expressed as mean ± standard error of mean. Tumor histology was confirmed by hematoxylin/eosin staining of formalin-fixed tissue.

Animal material

The mice experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of MGH.

Patient material

The experiments on patient specimens were approved by the local ethics committee of the Lund University, Lund, Sweden (Katja *et al.*, 2012).

Statistical analysis

Data from different experiments were represented as means ± standard deviation from at least three independent experiments. To analyze cell viability, linear regression analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Significance was established at $P<0.05$, as customary.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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