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## RESEARCH ARTICLE

# Multiple signaling pathways converge on proapoptotic protein BAD to promote survival of melanocytes

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## Abstract

Melanocyte survival is mediated by diverse signaling pathways. However, the molecular mechanisms they use and molecules that they target are incompletely understood. Here, we show that melanocyte survival is mediated by diverse, non-redundant signaling pathways, including ERK1/2, AKT, PKA, and PKC. Each of these pathways is exerting prosurvival effects by phosphorylating the BAD. While Ser112-BAD phosphorylation is regulated by pERK, pPKA and pPKC, Ser136 and Ser155 phosphorylation are exclusively controlled by pAKT and pPKA, respectively. Inhibition of these pathways individually resulted in only modest apoptosis; however, most significant apoptosis, as a result of BAD dephosphorylation, was seen when all pathways were inhibited concurrently. BAD phosphorylation was essential for survival of melanocytes as cells expressing phosphorylation-deficient BAD were not rescued by any of the identified pathway. Furthermore, melanocytes became insensitive to kinase inhibitor-induced apoptosis when BAD expression was knocked down by BAD-shRNA. Overexpression of BAD in melanocytes stimulated faster apoptosis in response to kinase inhibitors. Taken together, our results show that BAD is acting as a convergence point for diverse survival pathways in melanocytes. Understanding the molecular mechanisms of melanocyte survival provides fundamental new insights into physiological mechanisms involved in the development of various melanocyte pathologies such as melanoma and vitiligo.

## KEYWORDS

AKT, apoptosis, MAPK, PKA, PKC

## 1 | INTRODUCTION

Skin is the largest organ of the body and interfaces with the environment. Melanocytes are located in the basal layer of the epidermis and they are unique in synthesizing melanin pigment. Melanocytes are exposed to various environmental

and biochemical insults, such as pollutants, UV radiation, and endogenous reactive oxygen species such as hydrogen peroxide.<sup>1,2</sup> To sustain these cues, melanocytes deploy multicomponent signaling mechanisms that protect them from stressors.<sup>3</sup> Melanocyte survival and proliferation are controlled by a complex network of signaling mechanisms.

**Abbreviations:** AKT, protein kinase B; BAD, BCL2-associated agonist of cell death; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; shRNA, small hairpin RNA.

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Extracellular Signal-regulated Kinases (ERK1/2) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways are the most widely investigated mechanisms due to their role in survival and proliferation of normal melanocytes and melanoma cells.<sup>3-6</sup> While in normal melanocytes these pathways are modestly activated, in melanoma cells they are hyperactivated due to multiple genetic aberrations in the components of these pathways.<sup>7,8</sup> In addition to these pathways, melanocyte survival is also regulated by other mechanisms, such as BCL-2 family proteins.<sup>9,10</sup>

BCL-2 family is comprised of more than 20 members and grouped into three subfamilies according to their function: antiapoptotic (eg, BCL-2, BCL-XL, MCL-1), proapoptotic (eg, BAX, BAK) and BH3-only proapoptotic (eg, BAD, BIM) proteins. Most normal and cancerous cells express proapoptotic and antiapoptotic BCL-2 family proteins in a certain ratio, and through a complex interaction and based on their cellular compartmentalization, these proteins promote cell survival or apoptosis. It was shown that melanoma cells are extremely resistant to chemotherapeutics because of the hyperactivation of various survival signaling pathways and the aberrant expression of diverse BCL-2 family proteins.<sup>3,6-12</sup> Posttranslational modifications, such as phosphorylation, of certain BCL-2 family proteins also seem to play a major role in melanocyte survival.<sup>13-15</sup>

BAD is one of the proapoptotic proteins of BCL-2 family that, when de-phosphorylated, can bind to antiapoptotic proteins such as BCL-2 and BCL-XL and promote cell death. We previously showed that the ability of BAD to modulate apoptosis is controlled by mechanisms that regulate its phosphorylation state.<sup>16-18</sup> BAD can be phosphorylated at least at three different serine (Ser) residues, S112, S136, and S155. In phosphorylated state, BAD binds to 14-3-3 proteins and becomes sequestered in cytoplasm, and promotes cell survival, whereas in dephosphorylated state BAD induces apoptosis. Previous reports by us and others showed that growth factors, neuropeptides, and cytokines play a role in the survival of several cell types including melanocyte/keratinocyte, through modulation of BAD phosphorylation.<sup>14-19</sup>

Although previous studies have identified various survival pathways in melanocytes, their relative contribution to the melanocyte survival and their dependency on BAD phosphorylation remains incompletely understood. It was previously shown that BAD phosphorylation at S112 by MAPK pathway in melanocytes and melanoma cells correlates with survival.<sup>14,15</sup> However, BAD phosphorylation at other sites and the signaling mechanisms controlling BAD phosphorylation and its correlation with melanocyte survival were not investigated. Thus, a comprehensive investigation on the role of BAD and its phosphorylation in melanocyte physiology is still lacking. This prompted us to explore the potential role of BAD and signaling pathways that regulate BAD phosphorylation in melanocyte survival. In this paper, we demonstrate

that BAD is kept phosphorylated at multiple sites. Diverse signaling pathways partially regulate melanocyte survival by regulating BAD phosphorylation.

## 2 | MATERIALS AND METHODS

### 2.1 | Cells and reagents

Primary human epidermal melanocytes (PHE melanocytes), M254 medium, Human melanocyte growth supplement (HMGS), alamarBlue cell viability reagent were purchased from Life Technologies (Grand Island, NY, USA). Human epidermal melanocyte cell line transformed with hTERT (hTERT melanocytes) was bought from Applied Biological Materials Inc (Richmond, BC, Canada). PIG1 melanocyte cell line was a gift from Dr Caroline Le Poole, Northwestern University, Chicago, Illinois, USA. Protein G agarose beads and antibodies, unless specified, were purchased from Cell Signaling Technology (Danvers, MA, USA). JetPRIME transfection reagent, U0126 and H89 were procured from VWR (Piscataway, NJ, USA). Go6983 was from Selleck Chemicals. All other chemicals were from Sigma (Milwaukee, WI, USA). The pcDNA-HABAD-wt, pcDNA-HABAD-1SA, pcDNA-HABAD-2SA, and pcDNA-HABAD-3SA were as described by us previously.<sup>17</sup>

### 2.2 | Cell culture

Primary human epidermal melanocytes, PIG1 melanocytes, and hTERT melanocytes were cultured in M254 medium supplemented with HMGS growth supplement and Antibiotic-Antimycotic (Gibco) at 37°C in a humidified incubator of 5% CO<sub>2</sub>.

### 2.3 | Transfection and immunoprecipitation

Melanocytes were plated in 10 cm BioCoat cell culture plates. Transient transfection was performed when cells reached 70% confluence using jetPRIME transfection reagent using manufacturer recommended protocol. The total amount of transfected DNA was maintained at 6 µg (2.0 µg HA-BAD and 4.0 µg of empty pcDNA vector) in the 10-cm plate. In some experiments, cells were co-transfected with 1.0 µg of GFP vector to track the efficiency of transfection. Approximately, 20 hours after transfection, medium was replaced with supplement-free M254 medium and cells were treated with inhibitors where needed. Cells were harvested in cell lysis buffer, containing 20 mM Tris, pH 7.4, 1% Triton X 100, supplemented with protease and phosphatase inhibitors (Roche Diagnostics). The clarified cell

lysates were incubated with 6 µg of anti-HA antibodies (12CA5) overnight at 4°C, followed by protein G agarose beads for another 3 hours. Beads were washed three times with cell lysis buffer, and then, subjected to SDS-PAGE and Western blotting.

## 2.4 | Apoptosis and survival assays

To measure the apoptosis, cells were lysed in cell lysis buffer in the absence of phosphatase inhibitors and these lysates were used to assess apoptosis by measuring caspase-3 activity using the fluorogenic caspase substrate Ac-DEVD-AFC, as described.<sup>16-18</sup> For luciferase survival assays, cells were co-transfected with 0.1 µg luciferase vector and 0.5 µg of either empty vector or HA-BAD-wt, HA-BAD-1SA, HA-BAD-2SA, or HA-BAD-3SA in 6-well plates. The total amount of transfected DNA per well of 6-well plate was maintained at 1.5 µg with empty pCDNA vector. Where indicated some transfected cells were incubated with 20 µM of pan caspase inhibitor, Z-VAD-FMK, until cell lysis. About 20 hours after transfection, cells were washed with PBS, cell lysis performed, and luciferase activity was measured on a microplate luminometer as described by us previously.<sup>16</sup> In survival assays where GFP fluorescence was measured, cells were co-transfected as described for luciferase assay with the exception that luciferase vector was replaced with GFP vector. Cells were dislodged and placed in 96-well black plates with clear bottom and the GFP fluorescence was measured by fluorescence microplate reader (EnSight, PerkinElmer) with excitation at 485 nm and emission at 530 nm. Cell viability measurement using alamarBlue reagent was performed as suggested by manufacturer. In brief, melanocytes were seeded into 96-well plate, and approximately 20 hours later, cells were exposed to various inhibitors for 48 hours in 100 µL volume. Then, 100 µL of alamarBlue dye reagent was added to each well and incubated for 3 hours. At the end of incubation, the fluorescence was measured using a microplate reader with an excitation between 530 and 560 nm and an emission at 590 nm.

## 2.5 | RNA interference

HEK 293 cells were transfected with pLL3.7 vector containing either BAD-shRNA (BAD-shRNA-1) or a scramble-shRNA as reported by us,<sup>16,17</sup> in combination with packaging vectors (VSVG, RSV-REV, and pMDL g/p RRE). After 48 hours, supernatants were collected from these cells and used to infect PIG1 and PHEM cells. Forty-eight hours after infection, cells were analyzed by Western

blot to check for the BAD expression and plated for subsequent experiments. In a separate set of experiments, BAD knockdown was achieved using two independent BAD-shRNAs (BAD-shRNA-2 and BAD-shRNA-3) using “BAD Human shRNA Plasmid Kit” (OriGene Tech Inc, Rockville, MD, USA), using manufacturer recommended protocol.

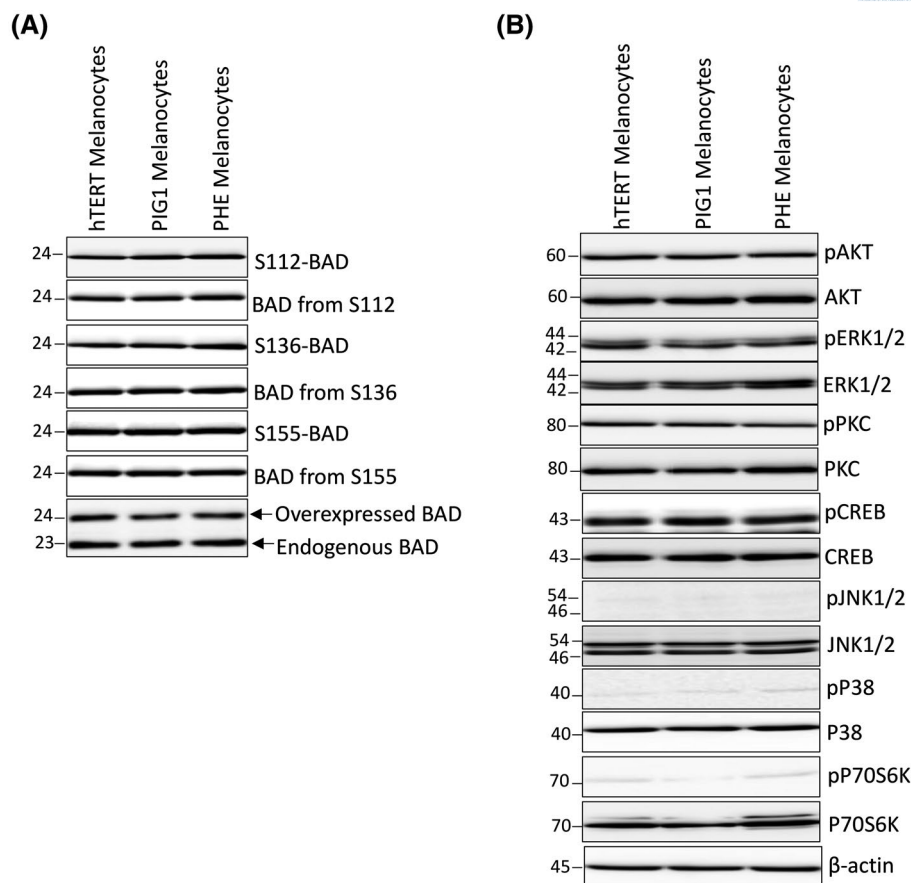
## 2.6 | Statistical analysis

All the statistical tests were two-sided. Unless otherwise stated, the error bars represent SD of the biologic triplicates. Differences between results were assessed for significance using the Student's *t* test. Differences were considered significant at *P* values ≤.05.

# 3 | RESULTS

## 3.1 | BAD is phosphorylated at multiple sites in melanocytes

Previously, we showed that proapoptotic protein BAD plays a dual role: when phosphorylated it inhibits apoptosis and in dephosphorylated state it induces apoptosis.<sup>16-18</sup> To address the role of BAD and its phosphorylation in melanocyte survival, we examined the expression and phosphorylation of BAD in PHE melanocytes, PIG1 melanocytes, and hTERT melanocyte cells. The latter two are immortalized human epidermal melanocyte cell lines which are frequently used in fundamental understanding of the molecular mechanisms in melanocytes.<sup>20,21</sup> BAD can be phosphorylated at three evolutionary conserved Serine residues such as S112, S136, and S155 of mouse BAD (corresponding to S75, S99, and S118 of human BAD), thereby inhibiting its proapoptotic function. While S112 BAD phosphorylation could be readily detected on endogenously expressed BAD using phosphorylation-specific antibodies, detection of S136, and S155 phosphorylation on endogenous BAD with respective phospho-antibodies was difficult due to insensitivity of these antibodies (Figure S1A). Thus, to overcome this limitation, we have ectopically expressed hemagglutinin-tagged BAD (HA-BAD) in melanocytes and BAD phosphorylation was detected on immunoprecipitated HA-BAD. Surprisingly, BAD is constitutively phosphorylated at S112, S136, and S155 in all melanocyte types, although the intensity of phosphorylation at different sites and cell types vary (Figures 1A and S1B). Our results also suggest that both endogenous and overexpressed BAD are similarly phosphorylated although the signal intensity on endogenous phosphorylation was weak (Figure S1A).



**FIGURE 1** A, BAD is constitutively phosphorylated in melanocytes: Indicated melanocyte cell types were cultured and transfected with HA-BAD and GFP to track the efficiency of transfection. About 20 hours after transfection, microscopic images were taken, cell lysates were collected and HA-BAD was immunoprecipitated using anti-HA 12CA5 antibodies. Phosphorylation-specific antibodies against phospho BAD S112, S136, and S155 were used to detect respective phosphorylation using western blotting. Each of the phospho-BAD blot was stripped and reprobed with total BAD antibodies. B, Diverse kinase are active in melanocytes: Melanocytes were cultured in M254 medium and when cells reached 70% confluence, cells were lysed and analyzed for activation of indicated kinases using respective phospho-specific antibodies. To judge the activation of PKA, antibodies that recognize S133-CREB phosphorylation was used. Each phospho blot was stripped and reprobed with antibodies that recognize respective total protein. Equal loading was verified using  $\beta$ -actin antibodies. Data are representative of at least three independent experiments

### 3.2 | Multiple kinases control BAD phosphorylation

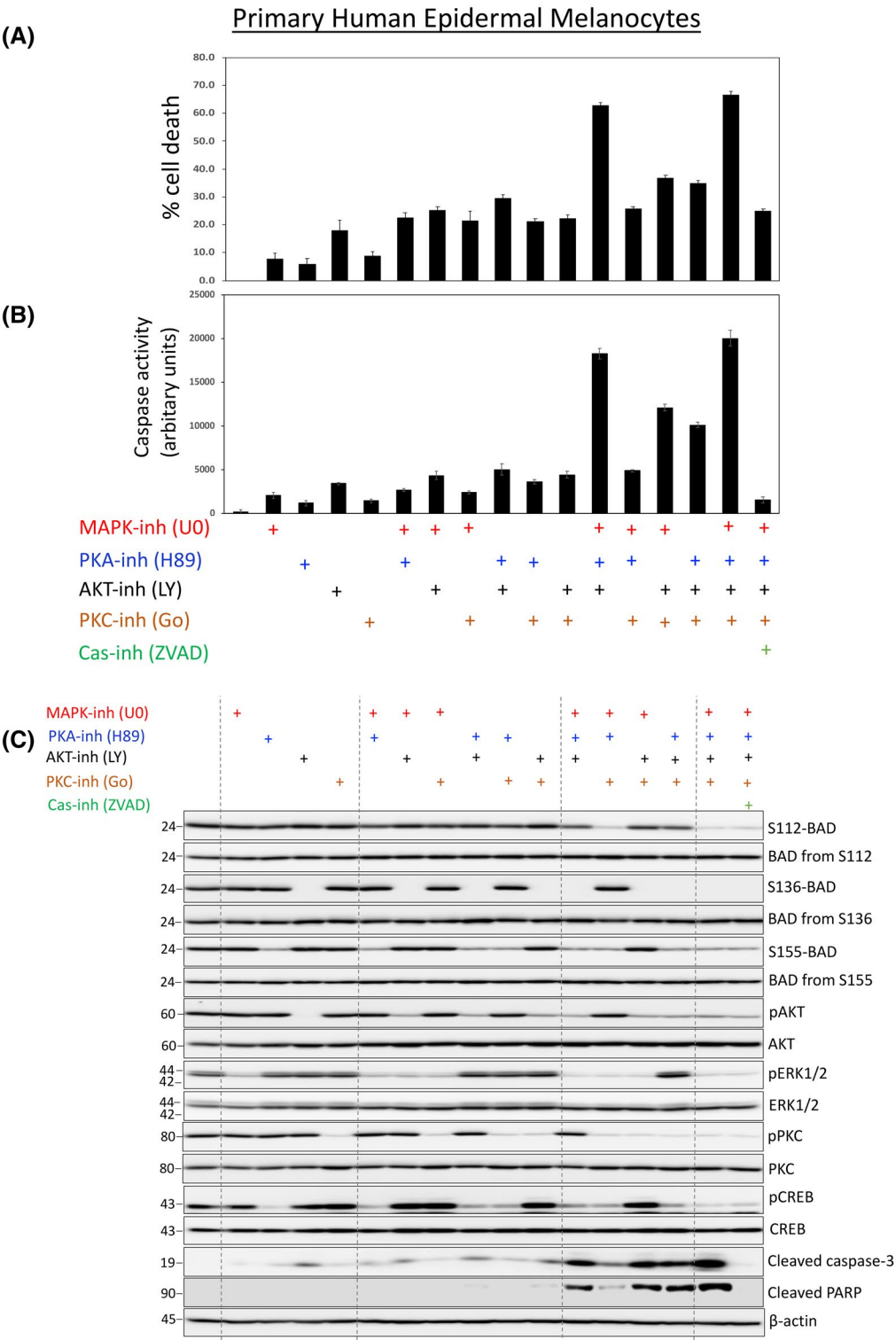
Since BAD was found to be simultaneously phosphorylated at all three different sites, we were interested to unveil the pathways that control BAD phosphorylation. To address this, we screened for the activation or phosphorylation of several kinases that have been previously shown to phosphorylate BAD either directly or indirectly in various cell types.<sup>16-18,22,23</sup> Thus, while S112 phosphorylation can be induced by ERK1/2, PKA, PKC, p38 or JNK; S136 is phosphorylated by AKT or p70S6 kinase. Protein Kinase A is a well-known kinase that can directly phosphorylate BAD at S155. Western blot analysis of cell lysates revealed that while ERK1/2, AKT, PKA, and PKC are activated in melanocytes as judged by their respective phosphorylation states, p38, JNK1/2, and p70S6 kinase are not active in these cells (Figure 1B).

Since the antibodies against PKA are not highly sensitive, we instead assayed CREB phosphorylation to judge the PKA activity. When PKA is activated, it phosphorylates its direct substrate CREB at S133,<sup>24</sup> which can be tracked using phospho-specific CREB antibodies. Together, these results suggest that ERK1/2, AKT, PKA, and PKC are constitutively activated in melanocytes and they may be responsible for inducing BAD phosphorylation and cell survival.

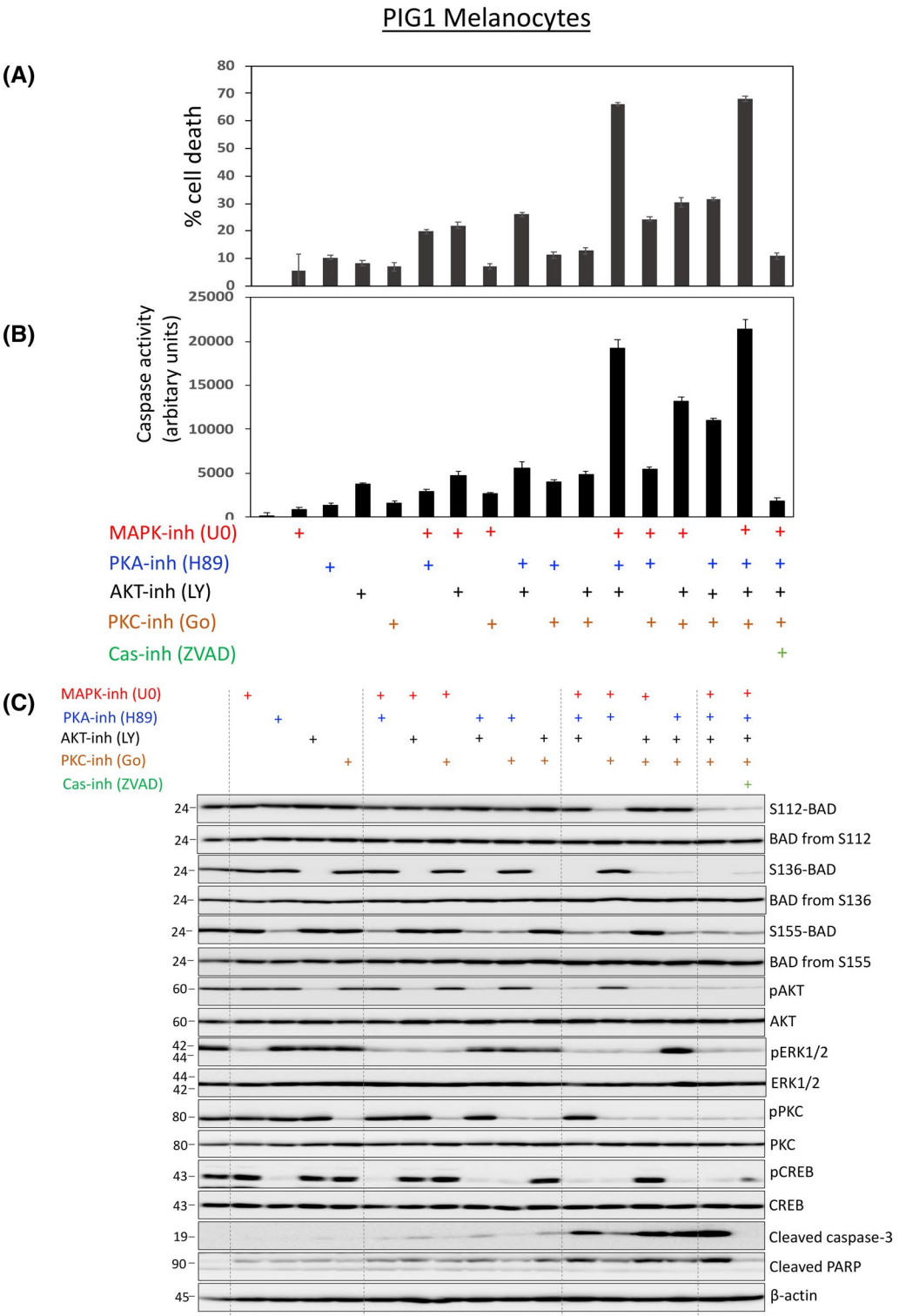
### 3.3 | BAD phosphorylation correlates with survival and dephosphorylation correlates with apoptosis

To address if the identified kinases have a role in melanocyte survival, we inhibited each of these kinases using pharmacological small molecule inhibitors and measured the cell death





**FIGURE 2** BAD phosphorylation correlates with survival. A, Primary human epidermal melanocytes were plated in 96-well plate and were exposed to 25  $\mu$ M LY294002, 10  $\mu$ M U0126, 10  $\mu$ M H89, or 1  $\mu$ M Go6983 and alamarBlue cell viability assay was performed 48 hours after addition of inhibitors. B, Cells were exposed to various inhibitors as in A and caspase activity in the cell lysates was measured using the fluorogenic substrate Ac-DEVD-AFC. For *P* values, please see Table S1. C, Primary human epidermal melanocytes were plated in 10 cm plates and transfected with HA-BAD as in Figure 1A and treated with various inhibitors as in Figure 2A. BAD phosphorylation was detected on immunoprecipitated HA-BAD as in Figure 1A. Whole cells lysates were used to probe for pAKT, pERK1/2, pCREB, pPKC, cleaved PARP, cleaved caspase-3, and  $\beta$ -actin. Phospho blots were stripped and reprobed with antibodies that recognize respective total protein. All experiments presented in this figure are representative of three independent experiments



**FIGURE 3** A, PIG1 melanocytes were treated as in Figure 2A and were subjected to alamarBlue cell viability assay. B, PIG1 melanocytes were treated as in Figure 2B and fluorogenic caspase assay was performed. For *P* values, please see Table S1. C, PIG1 melanocytes were transfected with HA-BAD and treated as in Figure 2C. BAD phosphorylation was detected on immunoprecipitated HA-BAD, and phosphorylation of kinases was detected using whole cell lysates. β-actin was used as loading control. Phospho blots were stripped and reprobed with respective total antibodies. All experiments presented in this figure are representative of three independent experiments

using various assays. We used U0126, LY294002, H89, and Go6983 to reduce the activities of pERK1/2, pAKT, pPKA, and pPKC, respectively. Each of these kinase inhibitors displayed specificity to its respective kinase, without any off-target effects at the concentrations used in this study (Figure S1C).

When melanocytes were incubated with each of these inhibitors individually, they induced cell death to an extent of 10% (Figures 2A, 3A, and Table S1). Further increase in cell death was observed when cells were treated with combination of inhibitors, and most significant cell death was noticed only when all four pathways were simultaneously inhibited. Concurrent treatment of cells with the four kinase inhibitors and a pan caspase inhibitor, Z-VAD-FMK, substantially rescued cells from apoptosis, pointing to the fact that the observed cell death is mainly mediated by apoptotic process. Similar results were obtained when cell death was measured by fluorogenic caspase activity assay (Figures 2B, 3B, and Table S1). To further corroborate these findings, we measured apoptosis by checking the cleaved products of caspase-3 and PARP, both considered as quantitative apoptotic markers, using the cell lysates by western blotting. Consistent with the caspase assay results, the highest levels of cleaved products of caspase-3 and PARP were noticed in cells treated with the combination of four kinase inhibitors (Figures 2C and 3C). Together, these results suggest that all four pathways are partially contributing to the survival of melanocytes.

To further clarify the role of BAD in melanocyte apoptosis, we addressed if a correlation exists between cell survival and BAD phosphorylation; and cell death and BAD dephosphorylation. Western blot analysis revealed that Ser112 phosphorylation was modulated by ERK1/2, PKA, and PKC pathways (Figures 2C and 3C). Interestingly, there was no significant reduction in S112 BAD phosphorylation when each of the pathways that control S112 phosphorylation, was individually inhibited by their respective inhibitors. Substantial dephosphorylation of S112 BAD was seen only when all three kinases were simultaneously inhibited. We also found that while S136 BAD phosphorylation was exclusively regulated by AKT, the phosphorylation at S155 was controlled by PKA alone. Simultaneous inhibition of all four kinases resulted in dephosphorylation of BAD at all sites and maximum apoptosis. Thus, there exists a strong correlation between melanocyte survival and phosphorylation of BAD; and apoptosis and BAD dephosphorylation (Figures 2 and 3), suggesting that active kinases in melanocytes deliver their cytoprotective effects through phosphorylation of BAD.

### 3.4 | Melanocytes display resistance to apoptosis induced by kinase inhibitors in the absence of BAD

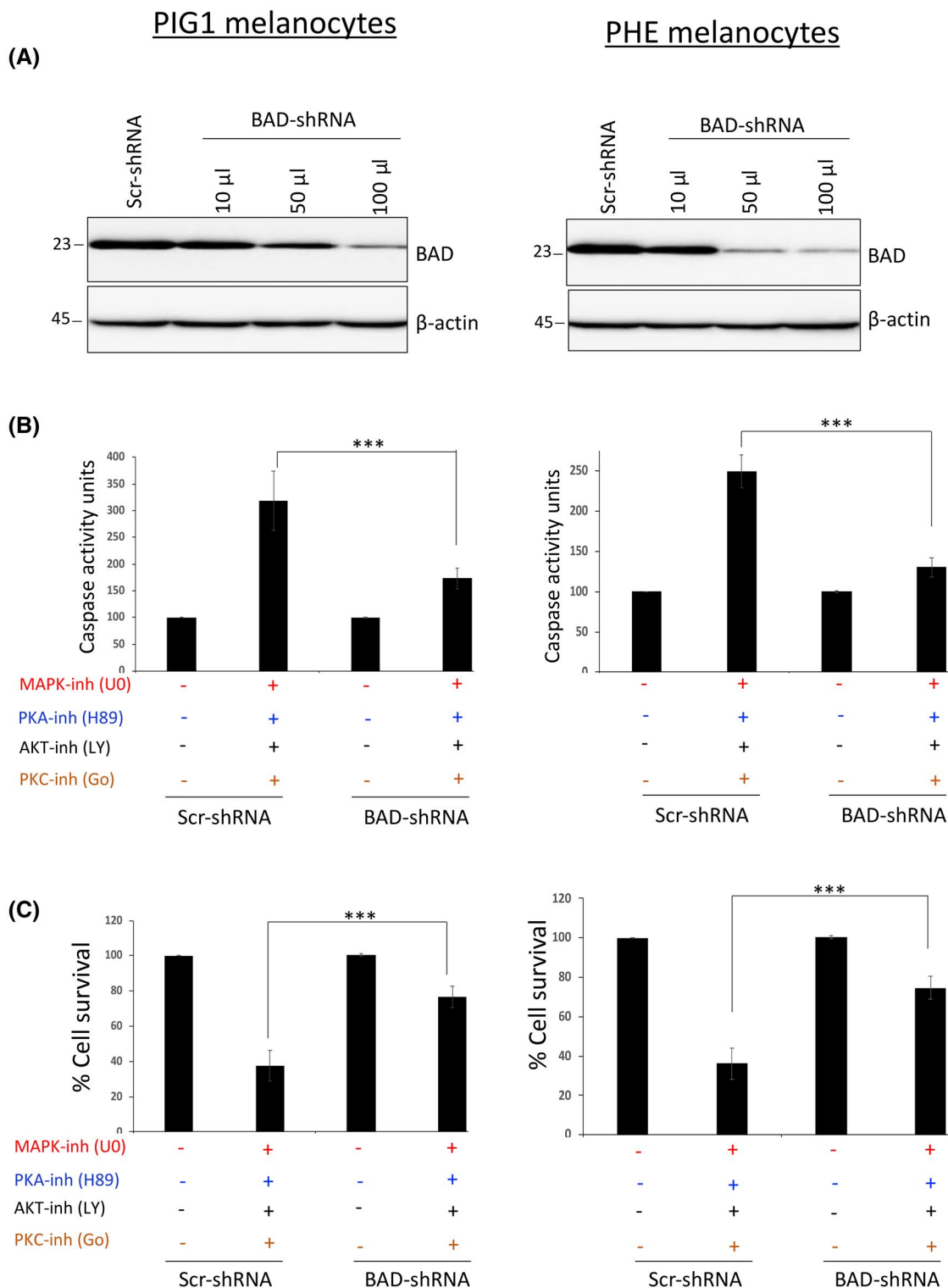
To establish that BAD expression is necessary for the induction of apoptosis by kinase inhibitors, we knocked down the endogenous BAD expression by delivering BAD-specific shRNA into melanocytes by lentivirus. These constructs also expressed GFP allowing us to track the transduction efficiency. The downregulation of BAD was progressive as

the quantity of lentivirus was increased. Substantial reduction in endogenous BAD expression was observed when cells were infected with 100  $\mu$ L of lentivirus (Figure 4A). At this viral load, the efficiency of infection was nearly 100% as judged by the expression of GFP (Figure S2A). Analysis of apoptosis, as measured by caspase activity assay, revealed that melanocytes expressing BAD-shRNA displayed a substantial reduction in apoptosis compared to cells expressing a scramble-shRNA, even in the presence of all four kinase inhibitors (Figure 4B). These results were further confirmed using an alternate cell death assay, the alamarBlue viability assay, and obtained similar results (Figure 4C). To rule out the possibility of off-target effects of our BAD-shRNA, we used two independent BAD-shRNAs (BAD-shRNA-2 and BAD-shRNA-3), targeting distinct regions of BAD to suppress expression. Consistent with the results shown in Figure 4, BAD knockdown with the alternate BAD-shRNAs dramatically reduced the expression of BAD and desensitized melanocytes to inhibitor-induced apoptosis. (Figure S2B,C). Together, our results suggest that the signaling pathways active in melanocytes such as MAPK, AKT, PKA, and PKC modulate their survival effects, at least in part, through BAD. Thus, BAD expression is necessary for inducing apoptosis or reducing survival in melanocytes.

### 3.5 | BAD phosphorylation is necessary for melanocyte survival

The above experiments did not directly address whether BAD phosphorylation is a prerequisite for melanocyte survival. Thus, to confirm that BAD phosphorylation is indeed critical for the survival of melanocytes, we made use of several phosphorylation-deficient mutant forms of BAD, where amino acid Serine residues have been mutated to Alanine residues, thereby mimicking a dephosphorylated form of BAD. Melanocytes were co-transfected with luciferase expression vector and BAD-wt (wild type), BAD-1SA (S112 to A112), BAD-2SA (S112 and S136 mutated to Ala), or BAD-3SA (all three Ser were replaced with Ala). Cell survival in these transfected cells was quantified by measuring the luciferase activity in the cell lysates. Previously, we and others have extensively used the luciferase assay to quantify the cell survival in transfected population of cells.<sup>16,17</sup> While cells expressing either empty vector or BAD-wt did not undergo significant apoptosis, those expressing BAD-1SA exhibited approximately 15% reduction in cell survival (Figure 5A). The decrease in cell survival was most apparent in cells expressing BAD-2SA and BAD-3SA even without treatment with kinase inhibitors. However, treatment of these mutant-BAD expressing cells with a pan caspase inhibitor, Z-VAD-FMK, rescued cells from apoptosis induced by mutant-BAD, and thus, survival was restored. These results were further confirmed using a different survival assay<sup>25,26</sup> which relies upon



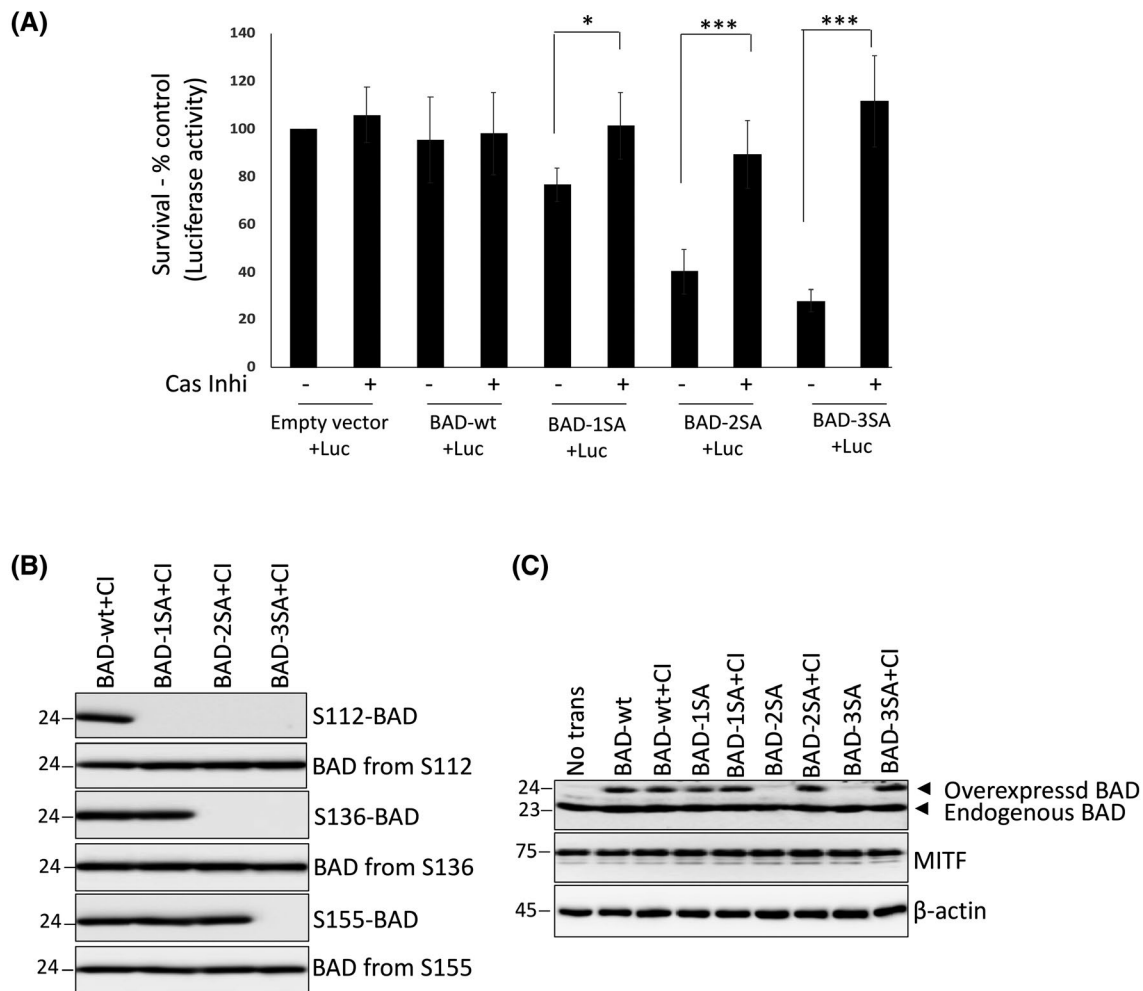


**FIGURE 4** BAD expression is required for inducing apoptosis. A, PIG1 (left panel) or PHE melanocytes (right panel) were infected with indicated quantity of lentivirus expressing BAD-shRNA or Scr-shRNA. About 48 hours after infection, cells were probed for BAD expression. B, Cells expressing either BAD-shRNA or Scr-shRNA were treated with the indicated inhibitors and caspase assay was performed using fluorogenic substrate Ac-DEVD-AFC. C, Melanocytes were seeded into 96-well plate and were exposed to the indicated inhibitors and cell death was measured using alamarBlue cell viability assay. Results are the average of three independent experiments  $\pm$  SD. The *P* values for the indicated comparisons were obtained by Student's *t* test. \**P* < .05, \*\**P* < .005, \*\*\**P* < .0005

measuring GFP fluorescence in cells co-transfected with mutant forms of BAD and GFP expression vector. Here, a substantial reduction in GFP fluorescence was noticed in cells

expressing BAD-2SA and BAD-3SA (Figure S3A). Figure 5B shows the western blot analysis of different forms of mutant-BAD. The phospho-specific antibodies were unable to detect

## PHE melanocytes



**FIGURE 5** BAD phosphorylation is necessary for melanocyte survival. A, Primary human epidermal melanocytes were co-transfected with luciferase vector and either empty vector, BAD-wt, BAD-1SA, BAD-2SA, or BAD-3SA. Where indicated cells were incubated with 20  $\mu$ M Z-VAD-FMK caspase inhibitor (CI). Approximately, 20 hours after transfection, cells were washed, lysed, and luciferase survival assay was performed. Results are the average of three independent experiments  $\pm$  SD. Statistically significant differences are indicated. The *P*-values for the indicated comparisons were obtained by Student's *t* test. \**P* < .05, \*\**P* < .005, \*\*\**P* < .0005. B, Primary human epidermal melanocytes were transfected with either HA-BAD-wt, HA-BAD-1SA, HA-BAD-2SA, or HA-BAD-3SA and then cells were incubated with 20  $\mu$ M Z-VAD-FMK. 20 hours after transfection, BAD phosphorylation was detected on immunoprecipitated HA-BAD. Failure to detect phosphorylation on HA-BAD mutant forms confirms the specificity of mutations. C, Cell lysates in A were probed with total BAD antibodies that recognize both endogenous and transfected BAD as well as with MITF antibodies.  $\beta$ -actin was used as loading control. Data are representative of three independent experiments

their respective phosphorylation on mutant BAD, demonstrating the specificity of mutations.

Western blot analysis of BAD expression in these cells showed that melanocytes can sustain the expression of BAD-wt and BAD-1SA even in the absence of Z-VAD-FMK (Figure 5C). However, the expression of BAD-2SA and BAD-3SA are lethal to cells, as they trigger cell death soon after their expression, and their cytotoxic effects can only be alleviated by treatment with caspase inhibitor. Together, these experiments confirm that BAD phosphorylation is necessary for survival of melanocyte. Previous studies showed that MITF-BCL2 pathway controls survival of melanocytes.

Since MITF expression levels were not altered in cells expressing mutant forms of BAD (Figure 5C), it is likely that BAD-mediated apoptosis is independent of MITF.

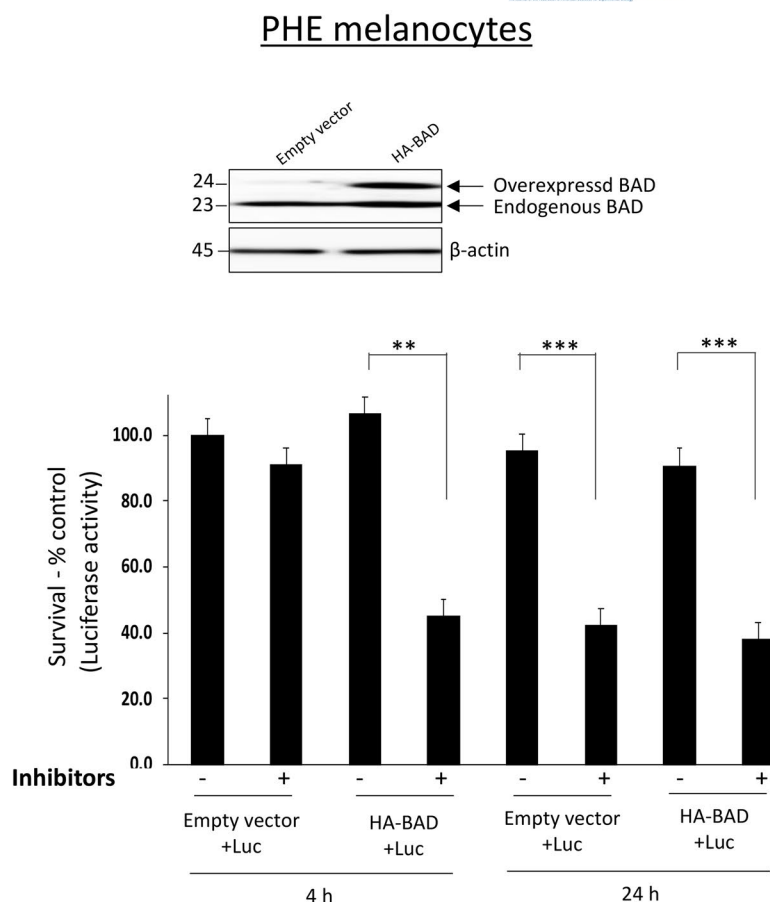
### 3.6 | Ectopic expression of HA-BAD induces early apoptotic death in melanocytes

As our previous results indicated that BAD plays a central role in the induction of apoptosis or survival of melanocytes, we then asked whether overexpression of BAD can induce early apoptosis in cells treated with kinase inhibitors. To address

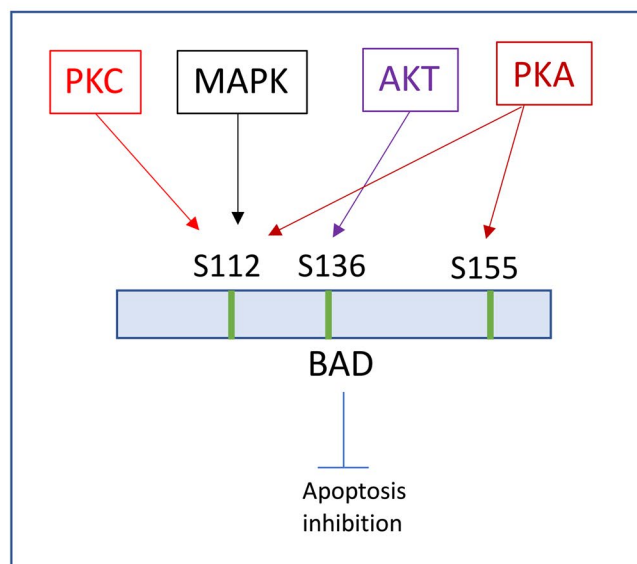
**FIGURE 6** BAD overexpression induces early apoptosis in melanocytes. A, Primary human epidermal melanocytes were co-transfected with luciferase vector and HA-BAD-wt. About 20 hours after transfection, cells were treated with indicated inhibitors for 4 and 24 hours. At the end of incubation, cells were lysed, and luciferase survival assay was performed. Expression of HA-BAD was shown.

Results are the average of three independent experiments  $\pm$  SD. Statistically significant differences are indicated. The  $P$  values for the indicated comparisons were obtained by Student's  $t$  test.  $*P < .05$ ,  $**P < .005$ ,  $***P < .0005$ . B, Survival signaling network in melanocytes: BAD plays the role of signaling node that integrates diverse cytoprotective signals in melanocytes. S112-BAD phosphorylation is controlled by MAPK, PKA, and PKC pathways. BAD phosphorylation at S136 and S155 are modulated by AKT and PKA, respectively

(A)



(B)



this, we transfected melanocytes with either empty vector or HA-BAD and a luciferase expression vector. Approximately, 20 hours after transfection, cells were treated with the cocktail of four kinase inhibitors and cell survival was measured at 4 and 24 hours using the luciferase assay. While melanocytes that expressed HA-BAD underwent apoptosis as early as 4 hours posttreatment, those that expressed empty vector did not undergo significant apoptosis at 4 hours (Figure 6A).

Both empty vector and HA-BAD expressing cells displayed similar levels of reduction in cell survival at 24 hours. Similar results were observed using GFP fluorescence survival assay (Figure S3B). Together, these experiments confirm that BAD phosphorylation plays a critical role in melanocyte survival.

In summary, our results show that ERK1/2, AKT, PKA, and PKC are vital kinases responsible for cytoprotective effects of melanocytes. Each of these pathways/kinases is

partially responsible for survival and they operate by inducing BAD phosphorylation to tightly regulate survival.

## 4 | DISCUSSION

Data presented in this study reveal that melanocyte survival is mediated by a complex network of BAD-dependent and BAD-independent pathways. Multiple kinases negatively regulate the activity of proapoptotic BAD by phosphorylating it at various serine residues. Thus, BAD is inactivated by several pathways, each inducing BAD phosphorylation at specific site, thereby contributing to the survival. These results suggest that regulation of melanocyte survival is more complex than previously described.

Initial observations had revealed a key role for BAD in regulating melanocyte biology. It has been shown that dopamine can induce the cell death of a mouse melanocyte cell line by increasing the BAD expression.<sup>27</sup> Furthermore, most previous studies in melanocytes or melanoma cells described the dominant role of a single pathway that regulate BAD phosphorylation at only one site. For instance, in cultured melanoma cells, S112 BAD phosphorylation was shown to be mediated by RAF/MAPK pathway, thereby contributing to cell survival.<sup>14,15</sup> A large genome wide association study has identified an association of SNP in BAD with vitiligo pathogenesis.<sup>28</sup> Despite this potential role of BAD in cell survival and disease pathogenesis, a comprehensive investigation on the role of BAD and its phosphorylation in melanocyte physiology is still lacking. Understanding survival signaling in melanocytes is crucial because this information will help us to delineate the pathways that go aberrant in various melanocyte pathologies such as melanoma and vitiligo.

Phosphorylation by protein kinases has been recognized as one of the critical mechanisms by which virtually every function of eukaryotic cells is regulated, including cell survival. Our experiments in this study found that in melanocytes four different survival signal transduction pathways are active: AKT signaling, MAPK signaling, PKA and PKC signaling pathways, each one is inducing inactivation/phosphorylation of BAD, thereby imparting survival.

The growth factors produced in the melanocyte microenvironment in vivo or their supplementation in in vitro culture conditions trigger the activation of ERK1/2 and PI3K/AKT, two signaling pathways largely involved in proliferation and survival of melanocytes.<sup>3-6</sup> Dysregulation of MAPK pathway caused by mutations in BRAF or NRAS can constitutively activate this pathway and has been shown to be responsible for development of >60% melanomas.<sup>7,8</sup> Vemurafenib, a BRAF-V600E inhibitor, has been approved by FDA for treatment of melanoma. However, the durability of response is limited to 6-8 months, as tumor cells develop resistance and progress to more advanced stages. In addition to MAPK pathway, loss of

tumor suppressor PTEN expression and subsequent hyperactivation of PI3K/AKT pathway synergizes with BRAF mutations that lead to the progression to metastatic disease.<sup>3,6-8</sup> In vitro, sustained activation of MAPK and AKT pathways can contribute to the melanocyte survival under nutrient depleted conditions.<sup>3-5</sup> Consistent with these findings, our results show that each of these pathways is activated in melanocytes: while the MAPK pathway induced S112 phosphorylation, AKT is exclusively responsible for phosphorylation of BAD at S136. Together, they both displayed synergistic survival effects, but either of these kinases induced survival of melanocytes in the absence of other.

In addition to the activation of MAPK and AKT signaling in melanocytes, we made a novel observation that both PKA and PKC contribute to melanocyte survival by phosphorylating BAD. Cyclic AMP (cAMP) signaling plays a significant role in normal melanocyte differentiation and melanin pigment synthesis.<sup>3,29</sup> Increases in levels of cAMP results in the activation of PKA which, in turn, phosphorylates its downstream substrate CREB transcription factor. Compared to normal melanocytes and primary melanoma, higher expression of PKA subunits has been reported in metastatic melanoma.<sup>29,30</sup> Our results show for the first time that activated PKA in melanocytes induced BAD phosphorylation at both S112 and S155, thereby controlling cell survival. This is the only kinase that induced BAD phosphorylation at two different Ser residues. Thus, our results provide a possible explanation for a recent finding showing that forskolin-induced PKA/CREB activation conferred resistance to ERK1/2 pathway inhibition in BRAF-V600E melanoma.<sup>31</sup> Thus, despite inhibition of ERK1/2, melanocyte survival is largely unaffected due to parallel activation of PKA/CREB pathway, which keeps the BAD phosphorylated at S112 and S155.

Another important signal transduction pathway that control growth of normal melanocytes is PKC signaling. PKC activating phorbol ester compounds such as TPA are the main components of growth supplements used for melanocyte cultures in vitro.<sup>3,32</sup> Alterations in the expression of various isoforms of PKC have been linked to the growth regulation of normal melanocytes and melanoma cells.<sup>29,33,34</sup> For the first time, our results show that PKC induces cell survival by phosphorylating BAD at S112. Thus, there exists a high level of redundancy in the signaling pathways that induce BAD phosphorylation at S112. While S112 phosphorylation is regulated by three mutually independent pathways such as MAPK, PKA, and PKC; S136 and S155 are regulated solely by AKT and PKA, respectively. Concurrent inhibition of all four pathways induced strong synergistic apoptotic effects, confirming the crucial, but subordinate, role of each of these pathways. Evidence from the xenograft studies that supports the role of PKA and PKC in several cancers including melanoma<sup>29-35</sup> warrants in-depth analysis of these mechanisms.

It is interesting that while a single pathway is sufficient to suppress BAD activity and induce cell survival, however, melanocytes employ diverse cytoprotective pathways. This can be explained by the fact that melanocytes/skin are in continuous contact with several environmental and biochemical insults, which can induce stress and subsequent melanocyte death. To sustain these insults, melanocytes need a backup/redundant signaling pathways when the dominant signals form one pathway is reduced. This may also explain the unusual ability of melanocytes to have very long lifespan and where they can survive for decades in the epidermis.<sup>3</sup> One of the key reasons for their prolonged lifespan is enhanced expression of BCL-2 family proteins such as BCL-2, which imparts them with resistance to apoptosis. Thus, our observations in this study reinforce these previous findings and provide additional mechanisms that melanocytes deploy to escape from cell death induced by various extracellular and intracellular stimuli.

Our results also point to the occurrence of BAD-independent survival mechanisms, because despite inhibition of four signaling pathways which dephosphorylate BAD at all three sites or in melanocytes that express phosphorylation-deficient BAD (BAD-3SA), significant component of cells displayed resistance to these effects, providing evidence for the pathways that are operating independent of BAD. In this context, it is worth mentioning some of previous reports showing that melanocyte survival is mediated by the expression of other BCL-2 family proteins. For example, a combination of inhibitors of MCL-1 and BCL-XL showed synergistic effect in killing melanoma cells.<sup>11</sup> Together, these results suggest that BAD-dependent and independent mechanisms play a role in melanocyte survival.

In vitiligo, melanocytes in the epidermis are selectively destroyed due to excess oxidative stress, leading to the absence of melanin pigment and consequent appearance of depigmenting skin in affected individuals. Our recent RNA-seq study in melanocytes that are subjected to oxidative stress revealed the downregulation of several prosurvival proteins and increased expression of diverse proapoptotic proteins, including BAD, thereby contributing to the elimination of stressed melanocytes.<sup>36</sup> However, since cell death is also controlled by posttranslational mechanisms as demonstrated in this study, no previous study, including ours, has attempted to investigate the significance of phosphorylation of BAD or other BCL-2 family proteins in stressed/vitiligo melanocytes. Although the results presented in this study suggest that BAD might have a role in the elimination of melanocytes in vitiligo, the importance of signaling pathways identified in this study and BAD phosphorylation in vitiligo melanocytes remains to be investigated.

In summary, melanocyte survival is regulated by multiple pathways, including ERK1/2, AKT, PKA, and PKC that lead to BAD phosphorylation. Each of these kinases is partially

responsible for cytoprotection through a redundant mechanism where they operate together to regulate the activity of BAD in order to tightly regulate cell survival. Thus, BAD acts as a key signaling node that integrates diverse cytoprotective signals in melanocytes (Figure 6B). Further studies aimed at increased understanding of survival pathways in normal melanocytes and their aberrant regulation in melanoma or vitiligo development will lead to the identification of new targets for clinical development of treatments.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

K.S. Sastry and A.I. Chouchane designed research, analyzed data, and wrote the paper. K.S. Sastry and W.N. Ibrahim performed research.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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