RIP1 Kinase Is an Oncogenic Driver in Melanoma


Abstract

Although many studies have uncovered an important role for the receptor-binding protein kinase RIP1 in controlling cell death signaling, its possible contributions to cancer pathogenesis have been little explored. Here, we report that RIP1 functions as an oncogenic driver in human melanoma. Although RIP1 was commonly upregulated in melanoma, RIP1 silencing inhibited melanoma cell proliferation in vitro and retarded the growth of melanoma xenografts in vivo. Conversely, while inducing apoptosis in a small proportion of melanoma cells, RIP1 overexpression enhanced proliferation in the remaining cells. Mechanistic investigations revealed that the proliferative effects of RIP1 overexpression were mediated by NF-kB activation. Strikingly, ectopic expression of RIP1 enhanced the proliferation of primary melanocytes, triggering their anchorage-independent cell growth in an NF-kB-dependent manner. We identified DNA copy-number gain and constitutive ubiquitination by a TNFα autocrine loop mechanism as two mechanisms of RIP1 upregulation in human melanomas. Collectively, our findings define RIP1 as an oncogenic driver in melanoma, with potential implications for targeting its NF-kB-dependent activation mechanism as a novel approach to treat this disease.

Cancer Res; 75(8); 1736–48. ©2015 AACR.

Introduction

Aberrant activation of survival signaling pathways plays an important role in cancer development, progression, and resistance to treatment (1). In melanoma, identification of activating mutations in BRAF as the major cause of constitutive activation of the MAPK pathway has led to successful development of mutant BRAF-specific inhibitors in the treatment of the disease (2). However, primary and acquired resistance, which is commonly associated with activation of other survival pathways, remains a major obstacle in the quest for curative treatment (3, 4).

Receptor-interacting protein kinase 1 (RIP1) mediates both cell survival and death signaling and is emerging as an important determinant of cell fate in response to cellular stress, in particular, to activation of death receptors such as TNFα receptor 1 (TNFR1; refs. 5–8). Upon TNFR1 stimulation, RIP1, along with other proteins, including TRADD, TRAF2, cIAP1, and cIAP2, is recruited to form prosurvival complex I (5, 9, 10). This results in stabilization of RIP1 through K63-linked polyubiquitination and/or linear ubiquitination of the protein carried out by TRAF2/cIAPs and linear ubiquitin chain assembly complex (LUBAC), respectively (8, 11–13). K63 polyubiquitin and linear ubiquitin chains linked to RIP1 serve as substrates for binding of the TAB2/TAB3/TAK1 complex and NEMO, leading to activation of NF-κB, which plays an important role in regulating many cellular processes (11, 12).

The expression of cIAPs is regulated by second mitochondria-derived activator of caspases (Smac), which is released from mitochondria upon activation of mitochondrial apoptotic signaling (14). Once in the cytosol, Smac binds to cIAPs, leading to their degradation (14). A number of small-molecule chemicals that function pharmacologically as Smac mimetic have been shown to similarly cause cIAP degradation (15, 16). When cIAPs are inhibited, RIP1 is deubiquitinated by the ubiquitin-editing enzyme A20 and the deubiquitinase cylindromatosis (17, 18). This switches the function of RIP1 to that of promoting apoptosis in complex IIa containing FADD and caspase-8 upon exposure to TNFα (6, 8). In this case, the kinase activity of RIP1 is essential for TNFα-induced apoptosis (8). However, if caspase-8 activation is limited, RIP1 recruits RIP3, leading to formation of complex IIIb causing programmed necrosis (necroptosis) in some types of cells (6, 9). Noticeably, TNFα can also trigger apoptosis independently of RIP1 when protein synthesis is inhibited (19, 20). This induction of apoptosis is inhibitable by cFLIP, but is not affected by inhibition of RIP1 kinase activity (21).

Many studies in recent years have primarily focused on the role of RIP1 in induction of cell death (5–7). In particular, the role of RIP1 in apoptosis and necrosis of various types of cancer cells in response to therapeutic agents is being increasingly reported (6, 7). However, RIP1−/− mice display extensive apoptosis in selected tissues and die at age 1 to 3 days (5), indicating that RIP1 has an important role in cell survival in a tissue type-specific manner. Indeed, recent evidence suggests that RIP1 is constitutively polyubiquitinated by cIAPs and interacts with TAK to facilitate cell...
RIP1 in Melanoma

Materials and Methods

Cell lines and human tissues

Human melanoma cell lines described previously were cultured in DMEM containing 5% FCS (23). Human melanocytes (HEMa-LP, HEMs-DP, and HEMn-MP) were purchased from Banksia Scientific and cultured as previously described (24). Human fresh melanoma isolates were prepared from surgical specimens according to the published method (25). Individual cell line authentication was confirmed using the AmpFISTR Identifiler PCR Amplification Kit from Applied Biosystems and GeneMarker V1.91 software (SoftGenetics LLC). A panel of 16 markers was tested, and each cell line had a distinct individual set of markers present (26). Tissue microarrays were constructed from formalin-fixed paraffin-embedded (FFPE) melanocytic tumour specimens retrieved from the Department of Tissue Pathology and Diagnostic Oncology at the Royal Prince Alfred Hospital, Australia (Supplementary Table S1). Studies using human tissues were approved by the Human Research Ethics Committees of the University of Newcastle and Royal Prince Alfred Hospital, Australia.

Antibodies and reagents

The antibody against RIP1 used in immunoprecipitation and antibodies against clAP1, clAP2, TNFαR1, and NEMO were purchased from Santa Cruz Biotechnology. The antibody against RIP1 used in Western blotting was from BD Biosciences. The antibody against RIP1 from Novus Biologicals. The blocking antibody against TNFα and recombinant human TNFα was from R&D Systems. The RIP1 kinase inhibitor necrostatin-1 (Nec-1), the Smac mimetic SM406, and the caspase inhibitor z-VAD-fmk were from Selleckchem. The NF-κB inhibitors PDTC and BAY-11-7082 were from Sigma-Aldrich. The NF-κB reporter Kit was from QIAGEN.

IHC

IHC staining and quantitation were performed as described previously (27). Briefly, antigen retrieval was performed in a pressure cooker for 30 seconds at 125°C. Antibody detection was performed using the Dako Envision HRP Detection system/DAB as per the manufacturer’s instructions. Slides were counterstained with Azure B to differentiate the melanin from the brown DAB immunolabeling.

Cell viability

A CellTiter-Glo Luminescent Cell Viability Assay kit was used according to the manufacturer’s instructions (Promega). Cells were seeded at 5 × 10^3/well in 96-well plates overnight before treatment as desired. CellTiter-Glo Reagent (100 μL) was added and incubated for 10 minutes. Luminescence was recorded by Synergy 2 multidetection microplate reader (BioTek; ref. 28).

Clonogenic assays

Cells were seeded at 2,000/well onto 6-well plates and allowed to grow for 24 hours followed by the desired treatment. Cells were

Figure 1. RIP1 is commonly upregulated in human melanoma. A, representative microphotographs of IHC staining of RIP1. Scale bar, 100 μm. B, quantification of RIP1 expression in melanocytic tumors. Data shown are mean immunoreactive score (IRS) ± SEM. *, P < 0.05, Kruskal-Wallis test. C and D, whole-cell lysates from melanocytes and melanoma cells were subjected to Western blotting. W, wild-type; M, BRAF^V600E or NRAS^Q61R mutation; P, primary melanoma; M, metastatic melanoma.
A

B

C

D

E

F

G

H

I

Tet.Ctrl
Tet.RIP1
Tetracycline
+ – – – + – – +

MM200
+ – – + – + – +

IgR3
+ – + + + + + +

75 kDa - 37 kDa -

RIP1/GAPDH

0.41 0.34 0.56 0.54 0.98

Mel-RM ME4405

RIP1 shRNA1
+ – – + – – +

RIP1 shRNA2
+ – – – – + – +

Ctrl shRNA
+ + + + + + + +

RIP1 shRNA1
+ – – + – – +

RIP1 shRNA2
+ – – – – + – +

Ctrl shRNA
+ + + + + + + +

Mut-RIP1 cDNA
+ – – + – – +

Number of colonies

0 100 200 300 400 500 600 700 800

BrdUrd incorporation (absorbance 450 nm)

0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5

BrdUrd incorporation (absorbance 450 nm)

0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5

BrdUrd incorporation (absorbance 450 nm)

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BrdUrd incorporation (absorbance 450 nm)

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BrdUrd incorporation (absorbance 450 nm)

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BrdUrd incorporation (absorbance 450 nm)

0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5

Number of colonies

0 100 200 300 400 500 600 700 800

Number of colonies

0 100 200 300 400 500 600 700 800

Number of colonies

0 100 200 300 400 500 600 700 800

Number of colonies

0 100 200 300 400 500 600 700 800
then allowed to grow for a further 12 days before fixation with methanol and staining with crystal violet (0.5% solution).

Cell proliferation assays

BrdUrd cell proliferation assays were carried out using an assay kit (Cell Signaling Technology) as per the manufacturer’s instructions. Briefly, cells were seeded at 5 × 10^4/well in 96-well plates overnight before treatment as desired. BrdUrd (10 μmol/L) was added and cells were incubated for 4 hours before BrdUrd assays were carried out. Absorbance was read at 450 nm using a Synergy 2 multidetection microplate reader (BioTek).

NF-κB reporter assays

NF-κB activity assays were performed using an assay kit (Qiagen) as per the manufacturer’s instructions (29). Briefly, cells transfected with the NF-κB activity reporter with or without cotransfection with indicated siRNAs or cDNAs were subjected to Dual-Luciferase Reporter Assay (Promega) after desired treatment.

Anchorage-independent cell growth

Cells (5 × 10^3) were seeded in 0.3% cell agar layer, which was on top of 0.6% base agar layer in 12-well culture plates. Cells were then incubated for a further 30 days at 37°C and 5% CO₂. Cell colony formation was then examined under a light microscope.

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation were carried out as described previously (30, 31). In brief, equal amounts of protein extracts were resolved by SDS-PAGE and analyzed by Western blot. Immunoblotting and immunoprecipitation were carried out as described previously (27).

Quantitative PCR analysis

Quantitative PCR (qPCR) was carried out as described before (30). Brieﬂy, the reaction was carried out for 40 cycles: 95°C for 15 seconds; 60°C for 1 minute. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1. Primers were used as listed in Supplementary Experimental Procedures.

Small interference RNA

The siRNAs were purchased from GenePharma. Transfection of siRNAs was carried out in Opti-MEM medium (Invitrogen) using Lipofectamine reagent (Invitrogen) according to the manufacturer’s transfection protocol (27).

Short hairpin RNA

The human RIP1 shRNA Kit (TG320591), human PTEN shRNA Kit (TG320498), and scramble shRNA Kit (TG300130), in which shRNA constructs are in retroviral GFP vector (pGFP-VRS), were purchased from Origene and were used according to the manufacturer’s protocols.

Plasmid vectors and transfection

The lentivirus-based inducible gene expression system (ViraPower HiPerform T-REx Gateway Vector Kit) was purchased from Invitrogen. The pCMV-Myc-RIP1 construct was from Origene. A mutant RIP1 construct containing four silent mutations within the RIP1 shRNA1 target sequence was also cloned into the pCMV-Myc vector. The kinase dead RIP1 (RIP1-K45A) construct was from Dr. Zheng Gang Liu (National Cancer Institute, Bethesda). The pCR-cFLIP construct was from Dr. H Nakano (Juntendo University School of Medicine, Tokyo, Japan). Plasmids were transfected as described previously (27).

Melanoma xenograft mouse model

Studies on melanoma xenografts were approved by the Animal Research Ethics Committee of Shanxi Cancer Hospital of China and were carried out as previously described (27). Briefly, cells (1 × 10^6) were injected s.c. into each flank of male athymic nude mice (Model Animal Research Centre of Nanjing University, China). Tumor growth was measured every 2 days using a caliper. Mice were sacrificed, and tumors were weighed at 30 days after tumour cell transplantation.

Statistical analysis

The statistical analysis was performed using JMP Statistics Made Visual software. The Student t test or Kruskal–Wallis test was used to assess differences between different groups. A P value less than 0.05 was considered statistically significant.

Results

RIP1 is frequently upregulated in human melanoma

We examined the expression of RIP1 by use of IHC in tissue microarrays constructed from 100 FFPE melanocytic tumors (Supplementary Table S1; ref. 27). The results revealed that RIP1 expression was frequently increased in melanomas compared with nevi (Fig. 1A and B and Supplementary Table S1). However, there was no significant difference in RIP1 levels between thin and thick primary, or between primary and metastatic melanomas (Fig. 1B; Supplementary Fig. S1 and Supplementary Table S1), suggesting that RIP1 is upregulated at early stages in the pathogenesis of melanoma.

We also examined RIP1 expression in a panel of melanoma cell lines, which were generated from melanomas of different stages and had varying status of the most common mutations in BRAF (BRAFV600E) and NRAS (NRASQ61R) (Fig. 1C). RIP1 was also commonly expressed at increased levels in the melanoma cell lines irrespective of their origins and genetic backgrounds in comparison with cultured melanocytes [pooled melanocytes...]

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**Figure 2.**

RIP1 promotes melanoma cell proliferation. A–C, cells with RIP1 stably knocked down were subjected to Western blotting (A), BrdUrd incorporation (B), or clonogenic assays (C; n = 3, mean ± SEM). *P < 0.05, Student t test. Scale bar, 1 cm. D and E, cells with RIP1 stably knocked down transfected with shRNA-resistant RIP1 (Mut-RIP1); DNA were subjected to Western blotting (D) and clonogenic assays (E; n = 3, mean ± SEM). *P < 0.05, Student t test. Scale bar, 1 cm. F and G, MM200 and IgR3 cells stably transduced with vector (Tet.Ctrl) or inducible RIP1 (Tel.RIP1) were treated with Tet (0 μg/mL). Cells were subjected to Western blotting (F) or BrdUrd incorporation assays (G; n = 3, mean ± SEM). *P < 0.05, Student t test. H and I, cells transfected with RIP1-K45A (kinase-dead RIP1) were subjected to Western blotting (H) or treatment with Nec-1 (30 μmol/L) followed by BrdUrd incorporation assays (I; n = 3, mean ± SEM).

*P < 0.05, Student t test.
RIP1 promotes melanoma cell proliferation

We focused on examination of the functional significance of RIP1 upregulation in melanoma cells. Although RIP1 knockdown did not cause melanoma cell death, it inhibited cell proliferation as shown in BrdUrd incorporation and clonogenic assays (Fig. 2A–C). Introduction of a construct expressing shRNA-resistant RIP1 cDNA reversed the inhibitory effect (Fig. 2D and E), demonstrating the specificity of the RIP1 shRNA and consolidating the role of RIP1 in regulation of melanoma cell proliferation. We introduced an RIP1-expressing construct into MM200 and IgR3 cells that expressed relatively low levels of endogenous RIP1 to establish sublines (MM200 RIP1 and IgR3 RIP1) conditionally expressing RIP1 in response to Tet (Fig. 2F). Induction of RIP1 reduced cell viability by approximately 10%, which could be reversed by the general caspase inhibitor z-VAD-fmk, indicative of induction of apoptosis (Supplementary Fig. S3A; ref. 32). Nevertheless, the remaining cells with RIP1 overexpression acquired increased proliferation rates (Fig. 2G). Of note, the RIP1 kinase inhibitor Nec-1 did not affect cell death caused by RIP1 overexpression (Supplementary Fig. S3A), consistent with previous reports that introduction of a kinase domain-defective form of RIP1 induced cell death to the similar extent as overexpression of wild-type RIP1 (6, 20). Indeed, introduction of a kinase-dead RIP1 mutant resulted in similarly reduced viability of MM200 and IgR3 cells (Supplementary Fig. S3B). Nevertheless, kinase-dead RIP1 retained the ability to promote melanoma cell proliferation irrespective of the presence of Nec-1 (Fig. 2I), indicating that RIP1-mediated promotion of melanoma cell proliferation is similarly independent of its kinase activity. In support, Nec-1 did not affect melanoma cell proliferation even in MM200 and IgR3 cells overexpressing RIP1 (Supplementary Fig. S3C).

RIP1 promotes melanoma cell proliferation through activation of NF-κB

As reported before, NF-κB was constitutively activated at high levels in melanoma cells compared with melanocytes (Supplementary Fig. S4; ref. 33). However, the levels of activation were reduced when RIP1 was knocked down (Fig. 3A and B). Consistently, phosphorylation of IκB at Serine 32/36 (p-IκB) is that marked for degradation was decreased (Fig. 3B; ref. 34). Introduction of the shRNA-resistant form of RIP1 reversed the inhibitory effect of RIP1 knockdown on activation of NF-κB (Fig. 3A), further verifying the role of RIP1 in NF-κB activation. Indeed, Tak1 and NEMO that are essential for RIP1-mediated NF-κB activation were coprecipitated with RIP1 in melanoma cells (Fig. 3C; ref. 35). Moreover, RIP1 overexpression increased NF-κB activation in MM200 and IgR3 cells (Fig. 3D).

The significance of NF-κB in RIP1-mediated promotion of melanoma cell proliferation was demonstrated by introduction of a nondegradable mutant of IκBα (IκBα-S32A/S36A; ref. 29), which inhibited melanoma cell proliferation even when RIP1 was overexpressed (Fig. 3E and F). Similarly, the NF-κB inhibitors PDTC and BAY-11-7082 also abolished RIP1 overexpression-triggered enhancement in melanoma cell proliferation (Fig. 3G; ref. 29). These results indicate that NF-κB activation plays an important role in RIP1-mediated promotion of melanoma cell proliferation. This was further supported by the finding that RIP1 knockdown could not reduce melanoma cell proliferation when IκBα was co-knocked down, which caused NF-κB hyperactivation (Fig. 3H–J). Furthermore, the levels of NF-κB activity appeared positively associated with the levels of RIP1 expression in fresh metastatic melanoma isolates (Figs. 1D and 3K).

RIP1 modulates melanoma growth

To investigate whether elevated RIP1 contributes to melanocyte transformation, we introduced the RIP1-expressing construct into HEMn-MP melanocytes (Fig. 4A). All its effect on melanoma cells, RIP1 overexpression reduced viability of melanocytes by approximately 10% in a caspase-dependent manner (Supplementary Fig. S5). Nevertheless, it increased the proliferation rate of the remaining cells. This was associated with increased expression of IκBα and reduction in its phosphorylation (p-IκBα S32/36; Fig. 4I). The role of RIP1 in promoting melanoma tumorigenesis was confirmed by transplanting MM200 cells stably overexpressing RIP1 into nu/nu mice (Fig. 4J). This demonstrated that RIP1 overexpression increased NF-κB activation and promoted melanoma growth in vivo (Fig. 4K–M).

Posttranslational modification is critical for upregulation of RIP1 in melanoma cells

In contrast with the common increase in the RIP1 protein, there were wide variations in RIP1 mRNA levels in melanoma cell lines from 3 different lines (HEMα-LP, HEMn-DP, and HEMn-MP) were used to simplify analysis as these melanocytes expressed similarly low levels of RIP1. (Fig. 1C and Supplementary Fig. S2). The elevated RIP1 expression was also evidenced in a panel of 21 fresh metastatic melanoma isolates by immunoblotting (Fig. 1D).
and fresh isolates (Fig. 5A and Supplementary Fig. S6A). In particular, some melanoma cell lines expressed even lower levels of the RIP1 mRNA relative to melanocytes (Fig. 5A). qPCR analysis of genomic DNA showed that one of 11 melanoma cell lines and one of 7 fresh melanoma isolates had copy-number gain of the RIP1 gene (Fig. 5B and Supplementary Fig. S6B), in keeping with the TCGA dataset showing that RIP1 copy-number gain occurred in 5.3% (20/375) of melanomas (www.cbioportal.org/public-portal). These data suggest that although DNA copy-number gain contributes to upregulation of RIP1 in some melanomas, posttranscriptional upregulation is a more commonly involved mechanism. In support, the turnover rates of the RIP1 mRNA remained comparable between melanoma and melanocyte cell lines regardless of its expression levels (Supplementary Fig. S7). Furthermore, the turnover rates of the RIP1 protein were prolonged in melanoma cell lines expressing higher levels of the protein (Fig. 5C and D).

We examined the ubiquitination status of RIP1 in Mel-RM and ME4405 cells that expressed relatively high levels of the protein. RIP1 was constitutively ubiquitinated with K63-linked ubiquitin chains as determined using an antibody specifically directed to K63-linked ubiquitin (Fig. 5E), suggesting that ubiquitination of RIP1 by cIAP1 and cIAP2 plays a role in its upregulation in melanoma cells. This was supported by the physical association of cIAP1 and cIAP2 with RIP1 as shown by coimmunoprecipitation assays (Fig. 5F), and was further consolidated by down-regulation of RIP1 when cIAP1, and to a lesser extent, cIAP2, was reduced by the Smac mimetic SM406, or when cIAP1 or cIAP2 was knocked down (Fig. 5G–I).

Autocrine TNFα contributes to upregulation of RIP1 in melanoma cells

In keeping with previous reports (36), the TNFα transcript was readily detected in melanoma cells (Fig. 6A). Moreover, the TNFα protein was found in supernatants of melanoma cell cultures, although the levels varied widely among melanoma cell lines (Fig. 6B). Nevertheless, melanoma cells expressed in general higher levels of TNFα mRNA than melanocytes (Fig. 6A). Similarly, melanoma cell culture media contained increased levels of its protein compared with melanocyte cell culture media (Fig. 6B). Strikingly, the addition of a blocking antibody against TNFα in melanoma cell cultures caused reduction in RIP1 ubiquitination and expression and reduced the amount of cIAP1 and cIAP2 coprecipitated with RIP1 (Fig. 6C–E), suggesting that autocrine TNFα is responsible, at least in part, for upregulation of RIP1 through regulating cIAP-mediated RIP1 ubiquitination. Indeed, TNFα knockdown also triggered reduction in RIP1 ubiquitination and expression and reduced the amount of cIAP1 and cIAP2 coprecipitated with RIP1 (Fig. 6F–H). Furthermore, the TNFα blocking antibody or TNFα knockdown reduced NF-κB activation and decreased proliferation, albeit moderately, in melanoma cells, which could be abolished by RIP1 overexpression (Fig. 6I and J and Supplementary Fig. S8).

RIP1 protects melanoma cells from TNFα-induced apoptosis

Although RIP1 knockdown did not impinge on cell viability, it conferred sensitivity of a proportion of melanoma cells to apoptosis induced by recombinant TNFα (Fig. 7A). This suggests that endogenous RIP1 plays a role in protecting melanoma cells from the toxicity of TNFα. Treatment with Nec-1 did not have a similar sensitizing effect (Fig. 7B), indicating that RIP1 protects melanoma cells independently of its kinase activity.

We tested whether TNFα-induced apoptosis in RIP1 knockdown melanoma cells could be inhibited by cFLIP overexpression. As shown in Fig. 7C and D, RIP1 knockdown did not sensitize Mel-RM cells stably overexpressing cFLIP to TNFα-induced apoptosis, suggesting that RIP1 inhibits TNFα-triggered cFLIP-inhibitable apoptosis in melanoma cells. Indeed, the levels of cFLIP are reduced in melanoma cells with RIP1 knocked down (Fig. 7E). Co-knockdown of 1kβα reversed RIP1 knockdown-mediated reduction in cFLIP and enhancement in TNFα-induced killing (Fig. 7E and F), indicating that RIP1 protects melanoma cells from apoptosis induced by TNFα through activation of NF-κB.

Discussion

Although RIP1 can mediate both cell survival and death signaling, many studies in recent years focused on its role in induction of apoptosis and programmed necrosis by various cellular stresses (6, 7, 20, 32). Indeed, similar to our finding that introduction of exogenous RIP1 triggers apoptosis in a proportion of melanoma cells and melanocytes, RIP1 overexpression has been shown to cause apoptotic cell death in many other cell types (32). These observations suggest that RIP1 may function primarily as an anti-survival protein and contribute to tumor suppression. However, we found in this study that RIP1 expression was frequently upregulated in melanoma cells, pointing to a role of RIP1 in favoring the pathogenesis of melanoma, and indicating that the expression of RIP1 must be under tight control so that its cellular level remains constantly below a threshold to ensure avoidance of apoptosis. Nevertheless, how RIP1 at high levels induces apoptosis in a selective population of melanoma cells and melanocytes remains unknown. This does not require the kinase activity of RIP1, as neither Nec-1 nor the kinase-dead mutant of RIP1 impinged on cell death. The death domain (DD) of RIP1 allows it to interact with other DD-containing proteins including FADD (6, 20), which can in turn recruit caspase-8. It is conceivable that RIP1 at high concentrations may trigger proximity-driven dimerization of caspase-8, thus leading to its activation and apoptosis (6, 8). On the other hand, cells that can survive RIP1 overexpression may possess intrinsic or acquired mechanisms that antagonize activation of caspase-8, thus protecting against apoptosis.

An important finding of this study is that RIP1 promotes melanoma cell proliferation and enhances melanoma growth. However, in contrast with previous reports in other types of cells (35, 37), inhibition of p53 does not appear to play a major role in RIP1-mediated proliferation of melanoma cells, as knockdown of RIP1 similarly inhibited cell proliferation in p53-null melanoma cells (ME4405 cells; ref. 38). In addition, RIP1 knockdown does not affect p53 expression in melanoma cells (35, 37). Moreover, RIP1 knockdown did not affect the expression of peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) that has been recently shown to play a role in RIP1-mediated suppression of p53 though regulating mitochondrial oxidative phosphorylation and glycolysis in lung cancer cells (data not shown; ref. 39). Although the mechanism responsible for this cell type–dependent involvement of p53 in RIP1-mediated regulation of cell proliferation remains to be addressed, it is known that wild-type p53 is often expressed at high levels but fails to exert an effective tumor suppressive role in melanoma cells (38).
Figure 4. RIP1 modulates melanoma growth. A and B, HEMn-MP melanocytes transduced with RIP1 cDNA were treated with BAY-11-7082 (1 μmol/L) for 72 hours followed by BrdUrd incorporation assays (A) or NF-κB reporter assays (B; \( n = 3 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. C–E, HEMn-MP melanocytes transduced with RIP1 cDNA were subjected to Western blotting (C) or anchorage-independent growth assays (D and E; \( n = 3 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. Scale bar, 0.5 mm. F, representative photographs of xenografts of Mel-RM cells with or without RIP1 stably knocked down. Scale bars, 5 mm. G, growth curves of xenografts of Mel-RM cells with or without RIP1 stably knocked down (\( n = 6 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. H, comparison of weight of xenografts of Mel-RM cells with or without RIP1 stably knocked down harvested at 36 days after s.c. injection of the cells into flanks of nu/nu mice. (\( n = 6 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. I, lysates of crude tumor tissues were subjected to Western blotting. J, representative photographs of xenografts of MM200 cells transduced with the vector alone or RIP1 cDNA (\( n = 6 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. K, growth curves of xenografts of MM200 cells transduced with the vector alone or RIP1 cDNA harvested at 36 days after s.c. injection of the cells into flanks of nu/nu mice (\( n = 6 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. L, comparison of weight of xenografts of MM200 cells transduced with the vector alone or RIP1 cDNA harvested at 36 days after s.c. injection of the cells into flanks of nu/nu mice (\( n = 6 \), mean ± SEM). \( \ast \), \( P < 0.05 \), Student t test. M, lysates of crude tumor tissues were subjected to Western blotting.
NF-κB is not involved in RIP1-mediated promotion of cell proliferation in some types of cells (5, 39). However, we found that NF-κB activation was critical for RIP1 to promote melanoma cell proliferation. This was demonstrated by (i) RIP1 knockdown resulted in decreased NF-κB activation, whereas melanoma cells that survived RIP1 overexpression displayed increased NF-κB activation; (ii) TAK1 and NEMO were physically associated with RIP1; (iii) inhibition of NF-κB activation abated enhancement in proliferation of melanoma cells overexpressing RIP; and (iv), hyperactivation of NF-κB eliminated RIP1 knockdown–mediated inhibition of cell proliferation. The mechanisms regulating cell type–dependent involvement of NF-κB remain to be clarified. Regardless, our results indicate that RIP1 is responsible, at least in part, for constitutive NF-κB activation that is commonly found at high levels in melanoma cells (29, 40).

Although RIP1 was expressed at elevated levels in melanoma cells, its mRNA levels varied widely in melanoma cells, and in some cases, were even lower than the levels in melanocytes. These findings promoted us to examine posttranscriptional mechanisms responsible for RIP1 upregulation. Indeed, RIP1 was constitutively polyubiquitinated with K63-linked ubiquitin chains, which is known to stabilize the protein (8–11, 13). Consistent with the established role of cIAPs in K63-linked polyubiquitination of RIP1 (9, 10), cIAP1 and cIAP2 were physically associated with RIP1. Because cIAP1s are transcriptional targets of NF-κB and RIP1 contributes to constitutive activation of NF-κB (9, 22, 41), it seems that a dynamic positive feedback loop of NF-κB–cIAPs–RIP1 maintains the high levels of RIP1 expression and NF-κB activation in melanoma cells (Figs. 1A–D and 3A).

**Figure 5.**

Posttranslational modification contributes to upregulation of RIP1 in melanoma cells. A, total RNAs from melanocytes and melanoma cells were subjected to qPCR analysis ($n = 3$, mean ± SEM). B, genomic DNA from melanocytes and melanoma cells were subjected to qPCR analysis ($n = 3$, mean ± SEM). C, whole-cell lysates from melanoma cells treated with cycloheximide (CHX; 1 μg/mL) were subjected to Western blotting. D, quantitation of the Western blot bands as shown in C. E and F, RIP1 immunoprecipitates from whole-cell lysates of cells were subjected to Western blotting. G, cells treated with the SM406 (1 μM) for 48 hours were subjected to Western blotting. H and I, cells transfected with cIAP1 (H) or cIAP2 (I) siRNA were subjected to Western blotting.
Figure 6. Autocrine TNFα contributes to RIP1 upregulation. A, total RNAs from melanocytes and melanoma cells were subjected to qPCR analysis (n = 3, mean ± SEM). P < 0.05, Student t test. B, TNFα expression in culture media of melanocytes and melanoma cells as measured by ELISA (n = 3, mean ± SEM). P < 0.05, Student t test. C and E, RIP1 immunoprecipitates from whole-cell lysates of cells treated with a TNFα blocking antibody (2 μg/mL) were subjected to Western blotting. D, whole-cell lysates from cells treated with a TNFα blocking antibody (2 μg/mL) for 48 hours were subjected to Western blotting. F, cells transfected with the control or TNFR1 siRNAs were subjected to Western blotting. G and H, RIP1 immunoprecipitates from whole-cell lysates of cells transfected with the control or TNFR1 siRNAs were subjected to Western blotting. I and J, cells transduced with RIP1 cDNA were treated with a TNFα blocking antibody (2 μg/mL) followed by NF-κB reporter assays (I) or BrdUrd incorporation assays (J; n = 3, mean ± SEM). P < 0.05, Student t test.
Melanoma cells have long been known to secrete TNFα, which promotes melanoma progression (42). Consistent with this, we found that autocrine TNFα played an important role in sustaining the NF-κB–cIAPs–RIP1-positive feedback loop in melanoma cells (Fig. 6). Because the production of TNFα is transcriptionally regulated by NF-κB (42), it appears that TNFα is an essential built-in apparatus of the NF-κB–cIAPs–RIP1 feedback loop, and plays a driving role in activation of these intracellular components. These results suggest that autocrine TNFα may be involved in intrinsic resistance of melanoma cells to treatment. Indeed,
TNFα receptor activation contributes to resistance of melanoma to therapeutic agents such as BRAF inhibitors through activation of NF-κB (43). Autocrine TNFα also contributes to cell proliferation and resistance to treatment in some other types of cancers (44).

In addition to autocrine TNFα, cancer cells are commonly exposed to microenvironments characterized by chronic inflammation associated with high levels of TNFα production (45). Circumvention of the cell death–inducing potential is essential for cancer cells to evade this detrimental effect of TNFα. We found that, despite its established role in TNFα-induced cell death when cIAPs are inhibited (20, 32), RIP1 played a role in protection of melanoma cells from apoptosis induced by TNFα through NF-κB activation and subsequent upregulation of cIAP1. Thus, cIAP1 is a major effector of the NF-κB-cIAPs-RIP1 feedback loop that protects melanoma cells from apoptosis induced by TNFα. Of note, although TNFα plus Smac mimetic induces apoptosis in many types of cells including melanoma cells (6, 46), we did not observe significant apoptosis in all but one melanoma cell line treated with the combination of TNFα and the Smac mimetic SM406. This is conceivably due to cell line–dependent effects of the treatment.

The role of RIP1 as an oncogenic regulator in the pathogenesis of melanoma is consolidated by the finding that RIP1 overexpression promoted proliferation and triggered anchorage-independent growth of melanocytes. Indeed, the significant increase in RIP1 expression in primary melanomas compared with nevi indicates that RIP1 upregulation is an early event during melanogenesis. Importantly, RIP1 upregulation is not associated with oncogenic activation of NRAS or BRAF, suggesting that it may cooperate with oncogenic mutations in NRAS or BRAF to promote the pathogenesis of melanoma, and that inhibition of the oncogenic activity of RIP1 may negatively regulate melanoma growth irrespective of mutations in NRAS/BRAF, which often underlies resistance of melanoma to treatment (47). Our finding that RIP1 promotes melanoma cell proliferation through NF-κB points to the possibility of targeting the intermediate domain of RIP1 that is required for NF-κB activation as a novel approach in the treatment of melanoma (5, 48). However, this has to be cautiously evaluated, as RIP1 is known to mediate cancer cell death induced by various agents (5–7). Furthermore, inhibitors that target the intermediate domain of RIP1 have to be carefully designed so that they do not impinge on the kinase activity of RIP1 as do currently available RIP inhibitors such as Nec-1 that inhibit RIP1-mediated cell death (8, 49).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Dr. Zheng Gang Liu (National Cancer Institute, Bethesda) for kinase-dead RIP1 plasmids and Dr. H. Nakano (Juntendo University School of Medicine, Japan) for cFLIP plasmids.

Grant Support

This study was supported by Cancer Council NSW [RG 13-15 and RG 13-04] and National Health and Medical Research Council (APP1026458), Australia.

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Received July 25, 2014; revised January 29, 2015; accepted February 1, 2015; published OnlineFirst February 27, 2015.


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*Cancer Res* 2015;75:1736-1748. Published OnlineFirst February 27, 2015.

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