RanBP3 Regulates Melanoma Cell Proliferation via Selective Control of Nuclear Export

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Chromosome region maintenance 1-mediated nucleocytoplasmic transport has been shown as a potential anticancer target in various malignancies. However, the role of the most characterized chromosome region maintenance 1 cofactor ran binding protein 3 (RanBP3) in cancer cell biology has never been investigated. Utilizing a loss-of-function experimental setting in a vast collection of genetically varied melanoma cell lines, we observed the requirement of RanBP3 in melanoma cell proliferation and survival. Mechanistically, we suggest the reinstatement of transforming growth factor-β (TGF-β)-Smad2/3-$p21^{Cip1}$ tumor-suppressor axis as part of the RanBP3 silencing-associated antiproliferative program. Employing extensive nuclear export sequence analyses and immunofluorescence-based protein localization studies, we further present evidence suggesting the requirement of RanBP3 function for the nuclear exit of the weak nuclear export sequence-harboring extracellular signal-regulated kinase protein, although it is dispensable for general CRM1-mediated nuclear export of strong nuclear export sequence-harboring cargoes. Rendering mechanistic support to RanBP3 silencing-mediated apoptosis, consequent to extracellular signal-regulated kinase nuclear entrapment, we observed increased levels of cytoplasmically restricted nonphosphorylated/active proapoptotic Bcl-2-antagonist of cell death (BAD) protein. Last, we present evidence suggesting the frequently activated mitogen-activated protein kinase signaling in melanoma as a potential founding basis for a deregulated post-translational control of RanBP3 activity. Collectively, the presented data suggest RanBP3 as a potential target for therapeutic intervention in human melanoma.

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INTRODUCTION

MEK-ERK signaling is central to melanoma cell biology (Flaherty et al., 2012). Even so, the approaches directly targeting this constitutively activated pathway through small molecule inhibitors have accomplished encouraging initial clinical responses, their long-term success has been confounded by an expansive array of acquired resistance mechanisms (Solit and Rosen, 2014).

Although cellular signaling pathways and the molecular perturbations in their component proteins have frequently been associated with various aspects of cancer cell biology (Hanahan and Weinberg, 2011), the altered protein subcellular distribution, especially a cytoplasmic buildup of various tumor suppressor proteins, has started to emerge as another significant mechanism in cancer pathogenesis and therapeutic resistance (Cagnol and Chambard, 2010; Jiao et al., 2008; Kau et al., 2004; Turner and Sullivan, 2008). For example, the aberrant cellular distribution of inhibitory cell cycle regulators (p53, $p21^{Cip1}$, and $p27^{Kip1}$), transcription factors (FOXO and NF-κB), and tumor suppressors (INI1/hSNF5 and BRCA1) has been associated with cancer progression (Kau et al., 2004).

With chromosome region maintenance 1 (CRM1) being the core component of the nuclear export machinery, a gene frequently overexpressed in cancer cells (Huang et al., 2009; van der Watt et al., 2009), including melanoma (Pathria et al., 2012), and a candidate whose expression is associated with poor disease prognosis (Noske et al., 2008; Shen et al., 2009), most nuclear export inhibition strategies have primarily focused on developing novel CRM1 inhibitors (Turner et al., 2014). These efforts have translated into highly efficacious and relatively non-toxic Selective Inhibitors of Nuclear Export (SINE) class of CRM1 inhibitors that have recently made their way into the clinical trials (Gerecitano, 2014).

Ran binding protein 3 (RanBP3), a protein characterized as a cofactor for CRM1-mediated nuclear export, has been shown to be involved in the early steps of CRM1-mediated

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Abbreviations: BAD, Bcl-2-antagonist of cell death; CRM1, chromosome region maintenance 1; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase 1/2; NES, nuclear export sequence; MAKP, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; RanBP3, ran binding protein 3; siRNA, small interfering RNA; TGF-β, transforming growth factor-β

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export, where it helps stabilize the ternary complex between RanGTP, CRM1, and the export cargo. Furthermore, a recent study reports RanBP3 as a crucial player downstream of the oncogenic mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase signaling pathways, thus possibly linking aberrant oncogenic signaling with deregulated nuclear export activity (Yoon et al., 2008).

Although the known functionality as a CRM1 cofactor and the associations with major oncogenic signaling pathways would argue for a potential role of RanBP3 in cancer biology, surprisingly, never has this proposition been systematically investigated. Therefore, in this study we tested the requirement of RanBP3 function in melanoma biology and evaluated its candidacy as a therapeutic target.

RESULTS

RanBP3 is required for melanoma cell proliferation and survival

To investigate the role of RanBP3 in melanoma cell biology, we adopted a loss-of-function approach. RanBP3 silencing utilizing small interfering RNA (siRNAs) suppressed melanoma cell proliferation (Figure 1a). Furthermore, RanBP3 silencing in an extended panel of genetically heterogeneous—encompassing virtually every major melanoma-associated molecular alteration (mut-*BRAF*, mut-*NRAS*, mut-*NF-1*, mut-/del-*CDKN2A* (p16INK4a), mut-/del-*PTEN*, and mut-*TP53*; Supplementary Table S1 online)—melanoma cell lines similarly compromised proliferation (Figure 1b and Supplementary Figure S1 online). An increase in annexinV and annexinV + propidium iodide positive cell fraction on RanBP3 silencing demonstrated apoptotic induction in all the tested cell lines (Figure 1c). Further indication of caspase-3 cleavage and pan-caspase inhibitor-mediated alleviation of cell death corroborated its apoptotic nature (Supplementary Figure S2a and b online). Notably, WM983B-BR melanoma cells that are resistant to BRAF-inhibitor PLX-4032 (cultured in growth medium comprising PLX-4032) also exhibited apoptotic cell death on RanBP3 silencing (Figure 1d). However, RanBP3 silencing in primary human skin fibroblasts failed to elicit apoptotic cell death (Figure 1e). Further underscoring the differential dependence of melanoma cells on RanBP3 function, RanBP3 silencing in telomerase reverse transcriptase-immortalized (Pmel/hTERT/CDK4(R24C)/p53DD) nontransformed primary human melanocyte lines genetically engineered to ectopically express either $BRAF^{V600E}$ (referred to as HMEL-B) or $NRAS^{G12D}$ (referred to as HMEL-N) did not induce significant cell death (Supplementary Figure S2c). These results underscore RanBP3's requirement in melanoma cell proliferation and survival, although it is dispensable in a noncancerous setting.

Interestingly, a closer look at MeWo cells depicted morphological features typical of senescence (Supplementary Figure S3a online). Loss of light refractivity, enlargement, and increased vacuolization were observed in a high percentage of cells. RanBP3 silencing resulted in a high percentage of cells staining positive for the established senescence marker, β-galactosidase, and suppression of phospho-retinoblastoma levels (Supplementary Figure S3b and c).

Altogether, these data suggest RanBP3 as a regulator of melanoma cell proliferation and survival.

RanBP3 negatively modulates the TGF-β-Smad2/3-$p21^{Cip1}$ growth suppressor axis

RanBP3 has previously been reported as a nuclear export mediator of Smad2/3 and, thus, a negative modulator of the TGF-β-regulated $p21^{Cip1}$/$p15^{INK4b}$-mediated tumor suppressive program in primary human keratinocytes (Dai et al., 2009). In line with this report, we also observed the requirement of RanBP3 function for Smad2/3 nuclear export in a melanoma setting (Supplementary Figure S4 online). Consistently, in the melanoma cells, RanBP3 knockdown also triggered an induction of $p21^{Cip1}$ levels, an effect that was further augmented by the presence of TGF-β (Figure 2a). Interestingly, however, no change in $p15^{INK4b}$ protein levels was observed (Figure 2a). Furthermore, an increase in the G1 cell cycle fraction (Supplementary Figure S5 online) supported the increased $p21^{Cip1}$ and suppressed phospho-retinoblastoma levels (Supplementary Figure S3c). Importantly, a concomitant $p21^{Cip1}$ silencing, partially, yet significantly, relieved the antiproliferative action of RanBP3 silencing (Figure 2b and c). Furthermore, transforming growth-factor receptor pathway inhibition partially overcame $p21^{Cip1}$ induction and the antiproliferative activity associated with RanBP3 silencing (Figure 2d and e).

These data demonstrate a conserved negative association between RanBP3 and the TGF-β-Smad2/3-$p21^{Cip1}$ growth suppressive axis in a transformed cellular context.

RanBP3 and CRM1-mediated nuclear export

Although RanBP3 is frequently referred to as a cofactor for CRM1-mediated nuclear export, a systematic interrogation of its requirement in the nuclear exit of established CRM1 substrates has never been undertaken. Furthermore, to gain a better understanding of the additional mechanisms underlying the antiproliferative and apoptotic outcome associated with RanBP3 targeting, we undertook a detailed functional assessment of RanBP3 involvement in the nuclear export of three (Survivin, p53, and MEK1) previously established CRM1 substrates (Henderson and Eleftheriou, 2000). Notably, survivin and p53 were additionally implicated as the mechanistic basis for CRM1 inhibition-mediated apoptosis (Pathria et al., 2012). As reported previously (Pathria et al., 2012), Leptomycin B-mediated CRM1 inhibition triggered survivin nuclear entrapment (Figure 3a). In contrast, RanBP3 silencing failed to elicit a change in the survivin cellular distribution (Figure 3b). As shown previously (Chan et al., 2010), after its nuclear entrapment, the suppression of survivin levels was evident (Figure 3c), whereas, consistent with its failure to trigger survivin nuclear entrapment, RanBP3 silencing also failed to mitigate its expression levels (Figure 3c).

p53 is exported in a CRM1-dependent manner, with CRM1 inhibition triggering its nuclear entrapment (Freedman and Levine, 1998), overcoming its cytoplasmic proteasomal degradation, and thus, inducing its protein levels (Figure 3d). Again, in contrast to CRM1 inhibition, RanBP3 silencing failed to influence the p53 subcellular distribution or protein expression levels (Figure 3e). In line with its dispensability for the nuclear export of survivin and p53, MEK1 nuclear export, while relying on CRM1 function (Figure 3f), showed indifference to RanBP3 function (Figure 3g).