

**Poly(ADP-ribose) polymerase family member 14 (PARP14) is a novel effector of the JNK2-dependent pro-survival signal in multiple myeloma.**

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**Abstract**

Regulation of cell survival is a key part of the pathogenesis of multiple myeloma (MM). JNK signaling has been implicated in MM pathogenesis, but its function is unclear. To elucidate the role of JNK in MM, we evaluated the specific functions of the two major JNK proteins, JNK1 and JNK2. We show here that JNK2 is constitutively activated in a panel of MM cell lines and primary tumors. Using loss-of-function studies, we demonstrate that JNK2 is required for the survival of myeloma cells and constitutively suppresses JNK1-mediated apoptosis by affecting expression of poly(ADP-ribose) polymerase (PARP)14, a key regulator of B-cell survival. Strikingly, we found that PARP14 is highly expressed in myeloma plasma cells and associated with disease progression and poor survival. Overexpression of PARP14 completely rescued myeloma cells from apoptosis induced by JNK2 knockdown, indicating that PARP14 is critically involved in JNK2-dependent survival. Mechanistically, PARP14 was found to promote the survival of myeloma cells by binding and inhibiting JNK1. Moreover, inhibition of PARP14 enhances the sensitization of MM cells to anti-myeloma agents. Our findings reveal a novel regulatory pathway in myeloma cells through which JNK2 signals cell survival via PARP14, and identify PARP14 as a potential therapeutic target in myeloma.

**Keywords:** PARP14, c-Jun N-terminal kinase (JNK), survival, multiple myeloma, apoptosis

## Introduction

Multiple myeloma (MM) is a neoplasm of terminally differentiated B cells, characterized by accumulation of clonal, long-lived plasma cells in the bone marrow (BM) as well as extramedullary sites.<sup>1</sup> Despite the significant progress that has been made in the identification of deregulated pathways, MM remains incurable. Further understanding of the biology of this disease is therefore needed in order to develop more effective therapies.<sup>2,3</sup>

Notable characteristics of myeloma cells include a low proliferative capacity and a weak apoptotic index *in vivo*.<sup>1,2</sup> Thus, pro-survival mechanisms play central roles in the accumulation of the malignant clone in MM.<sup>2,4,5</sup> Among the signaling pathways involved in the regulation of cell survival are members of the mitogen-activated protein kinase (MAPK) family, which includes three members: extracellular-regulated kinase (ERK1/2), Jun N-terminal kinase (JNK) and p38-MAPK signaling.<sup>6-8</sup> While the functions of ERK1/2 and p38-MAPK in MM have been defined in recent studies,<sup>9,10</sup> the role of JNK in the pathogenesis of the disease is unclear.

Signaling via JNK plays key roles in induction of apoptosis, cell proliferation, transformation and survival through the phosphorylation and regulation of its substrates.<sup>11</sup> As these cellular responses are important in tumorigenesis, JNK signaling has been implicated in human cancers.<sup>11,12</sup> Two major JNK proteins, JNK1 and JNK2, are expressed ubiquitously and each has two different splicing isoforms (p46 and p54).<sup>11</sup> Although JNK1 and JNK2 have been considered redundant kinases, there is evidence for distinct or even opposing functions of JNK1 and JNK2 in tumorigenesis.<sup>12-15</sup> Thus, it has been reported that JNK2, but not JNK1, is essential for growth of various human cancer cells including lung,<sup>16,17</sup> glioblastoma,<sup>18,19</sup> prostate and skin.<sup>20-22</sup> In contrast, a recent study reported that JNK1 is essential for liver carcinogenesis, while JNK2 appears to be dispensable.<sup>12,23</sup> Taken together, these data infer a

context- and tissue-specific functions for JNK1 and JNK2 in cancer development.<sup>12</sup> Although it has been suggested that differential regulation of specific cellular targets is the molecular basis for the distinct functions of JNK1 and JNK2 in different tumours,<sup>12</sup> there is a paucity of understanding regarding the identification of targets regulated by the individual JNK members.

A role for JNK signaling in MM has also been reported, although the proposed functions are controversial.<sup>24-27</sup> Whilst JNK activation was shown to promote cell-cycle progression in MM,<sup>24</sup> JNK activation mediates apoptosis induced by treatment with anti-myeloma agents, often through activation of caspases.<sup>25-27</sup> Furthermore, inhibition of JNK with the chemical inhibitor, SP600125, induces growth arrest in MM cell lines.<sup>24</sup> However, the individual contribution of JNK1 and JNK2 to the pathogenesis of MM is not known due to the lack of chemical inhibitors specific for each JNK protein.<sup>28</sup>

Here we show that JNK2 is constitutively active in myeloma cells, and inhibits the pro-apoptotic activity of JNK1 through effects on poly(ADP-ribose) polymerase (PARP)14, thus promoting survival in myeloma cells. PARP14, a member of the macro-PARP subfamily of PARP proteins, is an ADP ribosyltransferase that transduces survival signals in murine primary B cells by regulating expression of B-cell survival factors as well as by repressing an apoptotic program involving caspases.<sup>29,30</sup> In the mouse, PARP14 has been shown to facilitate B-lymphoid oncogenesis driven by oncogenic c-Myc.<sup>31</sup> However, the mechanisms underlying the survival function of PARP14 have not been previously investigated in MM or other types of human cancer.

We demonstrate that PARP14 acts as a physiological downstream effector of the JNK2-dependent pro-survival signal by binding to JNK1 and inhibiting its activity. We also find that expression of PARP14 correlates with disease progression and poor prognosis in MM. Therefore, this study describes a novel regulatory pathway in myeloma cells through which

JNK2 promotes survival and suggests that selective inhibition of PARP14 might be of therapeutic value.

## Results

### JNK2 is constitutively active in myeloma cells

Activation of JNK in BM trephine biopsies from four patients with newly diagnosed MM was assessed by immunohistochemistry using an antibody that recognizes both phospho-active JNK1 and JNK2 (p-JNK). All myeloma cells showed strong nuclear staining for p-JNK in all the samples examined. In contrast, no p-JNK staining was detectable in the normal plasma cells in tonsillar samples (n=3) (Figure 1A). Consistently, analysis of JNK activation by immunoblotting in six additional MM cases showed considerable increase of p-JNK in BM plasma cells (selected as CD138<sup>+</sup>CD38<sup>hi</sup>CD45<sup>low/-</sup>)<sup>32</sup> in 5 out of 6 cases compared to normal B cells (Figure 1B, left). Interestingly, we also observed that intensity of p-JNK2 (p-p54, which is the prominent spliced form of JNK2) was significantly higher than p-JNK1 (p-p46, the prominent spliced form of JNK1)<sup>11</sup> (Figure 1B, right), indicating that JNK2 activity was increased in myeloma plasma cells. Increased levels of p-JNK2 were also found in a panel of MM cell lines compared to p-JNK1 (Figure 1C). The differences in the levels of p-JNK2 versus p-JNK1 in MM cell lines and primary tumors were not due to differential protein expression (Figures 1B,C). Further validation was obtained using a JNK2 kinase assay. A dramatically elevated JNK2 activity was detected in MM cell lines, whereas normal B cells lacked a comparable JNK2 activity (Figure 1C). In contrast, JNK1 kinase assay showed that JNK1 activity was nearly undetectable in all MM cells tested, and comparable to that found in normal B cells (Figure 1C). These data indicate that JNK2 is constitutively activated in myeloma plasma cells.

To establish the function of constitutive JNK2 activity in myeloma cells, expression of JNK2 was knocked down in the human MM cell line, RPMI-8226, using JNK2-specific shRNA lentiviruses. We used the pLL3.7 lentiviral vector to express JNK2 shRNA (shJNK2) together with GFP in a bicistronic fashion.<sup>33,34</sup> Lentiviruses expressing JNK1 shRNA (shJNK1) or non-specific shRNA (shNS) were used as controls. Knockdown was specific, as shRNAs did not affect expression of other related MAPK proteins (ERK1/2 and p38), as verified by western blots (WB) (Figure 1D). As shown in Figure 1E, shJNK2 expression caused a progressive reduction of the GFP-positive cells over time compared to shJNK1 or shNS, despite the three lentiviruses each yielding GFP-positive cells in RPMI-8226 cells with comparable efficiency two days after infection (supplemental Figure S1A). This demonstrates that cells expressing shJNK2 and GFP were negatively selected from the population due to cell death and indicates a role for JNK2 but not JNK1 in RPMI-8226 cell viability. In contrast, BJAB cells, which are derived from Burkitt's lymphoma (BL) tissue, were unaffected by infection with shJNK1, shJNK2 or shNS lentiviruses (Figure 1F) despite JNK1 or JNK2 protein being effectively knocked down (Figure 1G). These results are consistent with other studies demonstrating redundant roles for JNK1 and JNK2 proteins in BLs.<sup>14,35</sup>

### **Knockdown of JNK2 induces apoptosis**

To provide further evidence for cell death in shJNK2-infected MM cells, we quantified apoptosis measuring the sub-G1 population using propidium iodide (PI) staining as well as AnnexinV/7AAD staining and activation of caspases. Flow cytometric (FCM) analysis of PI-stained RPMI-8226 cells as early as 4 days after infection revealed that JNK2 silencing resulted in an approximate 6-fold increase in apoptosis, compared with JNK1 or control silencing (Figure 2A; supplemental Figure S1B). The percentage of apoptotic cells in shJNK2-infected RPMI-8226 cells further increased with time, and by 8 days post-transduction had reached 89.8% (Figure 2A; supplemental Figure S1B). We extended these analyses to two additional MM cell lines, MM1.S and JJN3. Efficient knockdown of JNK2 correlated with a

significant induction of apoptosis (Figures 2A,B; supplemental Figures S1C,D). Depletion of JNK2 also resulted in a robust cleavage of procaspase-9, -3 as well as of downstream PARP1, and the appearance of active cleavage products of the expected size in each case (shJNK2-infected cells). As expected, in BJAB cells JNK2 depletion did not induce apoptosis (Figure 2A; supplemental Figure S1F), confirming that JNK2 is not essential for basal survival of BJAB cells. In contrast to JNK2 silencing, the silencing of JNK1 did not affect survival in any of the cell lines tested (Figures 2A,B). Together, these data indicate that JNK2 is required for myeloma cell survival.

### **JNK2 constitutively inhibits JNK1-dependent apoptosis in MM cells**

As previous studies have shown that JNK2 negatively regulates JNK1 activity and promotes survival in colorectal cancer cell lines by constitutively suppressing JNK1-mediated apoptosis,<sup>36,37</sup> we investigated whether this was the case for MM. In-vitro kinase assays revealed that JNK2 knockdown led to a robust increase in JNK1 activity in MM cells compared to control shNS-infected cells (Figure 2C, JNK1 K.A.). This effect of JNK2 knockdown was specific, as p38 and ERK1/2 phosphorylation was comparable in shNS-, shJNK1-, and shJNK2-infected cells (Figure 2C). Notably, JNK2 depletion did not result in a significant increase of JNK1 activity in BJAB cells (supplemental Figure S1F, compare lanes NS and JNK2). As predicted, in all cell lines tested JNK2 activity was unaffected by JNK1 depletion (Figure 2C; supplemental Figure S1F, JNK2 K.A, compare lanes NS and JNK1). The data indicate that JNK2 specifically inhibits basal JNK1 activity in MM cells.

Next, we examined whether JNK1 silencing can rescue JNK2-depleted cells from apoptosis. As shown in Figures 2D and 2E, apoptosis induced by JNK2 depletion was completely reversed by co-silencing JNK1 with JNK2. Remarkably, cleavage of caspase-3 was abolished when JNK1 was co-silenced with JNK2 (Figure 2F), indicating that JNK1 is an essential

mediator of apoptosis induced by JNK2 depletion. Our data therefore indicate that JNK2 regulates myeloma cell survival by suppressing basal JNK1-mediated apoptosis.

### **JNK2 regulates expression of PARP14 in MM cells**

To dissect the mechanism of JNK1 inhibition by JNK2 in myeloma cells, we screened for known B-cell survival factors responsive to JNK2 silencing. PARP14 is a well-established pro-survival protein that has been implicated in protecting B-lymphocytes against apoptosis.<sup>30</sup> PARP14 expression has been also shown to promote B-cell lymphomagenesis through supporting cell survival signals in mice.<sup>31</sup> These observations led us to investigate whether levels of PARP14 were affected by JNK2 depletion. Indeed, PARP14 expression levels were markedly reduced in MM cells by knockdown of JNK2 but not JNK1 or control (Figure 3A). The reduction in PARP14 levels was specific, as there was no difference between shJNK1-, shJNK2-, and shNS-infected cells in terms of PARP9, another macro-PARP family member.<sup>38</sup> This indicates that JNK2 modulates PARP14 expression in myeloma cells (Figure 3A). Intriguingly, reduced PARP14 protein levels were not observed in JNK2-silenced BJAB cells, suggesting that PARP14 expression is controlled by cancer cell-specific mechanisms (Figure 3A).

To examine the expression of PARP14 further, BM biopsies from four MM patients were examined by immunohistochemistry. Over 80% of the neoplastic cells in all myeloma samples tested had high expression of PARP14. Normal control sections (n=3) showed very low PARP14 expression in a proportion of plasma cells within the tonsillar tissue. As with p-JNK, PARP14 expression was stronger in the nucleus as compared to the cytoplasm (Figure 3B). The expression of PARP14 was also confirmed by immunoblotting using MM cell lines and plasma cells obtained from the BM aspirates of six additional MM patients. PARP14 levels were increased in MM lines (sensitive to JNK2 depletion) and primary cells compared with normal B cells (Figure 3C). In contrast, 2 of 3 JNK2-independent BL cell lines (data not

shown), Ca46 and Raji, did not have detectable PARP14 protein (Figure 3B). The third cell line, BJAB, had high levels of PARP14 protein (Figure 3B), confirming that JNK2-independent mechanisms of regulation exist. To gain additional clinical insights, we examined the expression of PARP14 transcripts in MM and BL using publicly available gene-expression datasets.<sup>39-41</sup> We observed that PARP14 expression significantly increases in MM plasma cells compared to monoclonal gammopathy of undetermined significance (MGUS) or normal BM plasma cells, indicating a positive correlation between PARP14 expression and disease progression (Figure 3D, left). In contrast, no significant differences were observed between normal germinal center (GC) B-cells and BL cases (Figure 3D, right).

### **Knockdown of PARP14 induces apoptosis**

We next asked if PARP14, like JNK2, is required for MM cell survival. As with JNK2, depletion of PARP14 by shRNA (shPARP14) induced apoptosis (Figures 4A-C). Silencing was specific, as shPARP14 did not affect expression of PARP9 (Figure 4D). The impact of PARP14 knockdown in two additional MM cell lines, OPM-2 and KMS12BM, was monitored by the progressive disappearance of GFP-positive cells in shPARP14-infected cells (Figure 4E). Interestingly, PARP14 knockdown markedly increased JNK1 activity with magnitude similar to JNK2 knockdown, indicating that PARP14 is required for JNK1 inhibition (Figure 4C, supplemental Figure S2A). This effect of PARP14 knockdown was specific, as p38 and ERK1/2 phosphorylation/activation was comparable in shNS- and shPARP14-infected cells (supplemental Figure S2B). Similarly, JNK1 activation and a dose-dependent apoptosis were also induced by the pan-PARP inhibitor PJ-34, which has been previously used to examine PARP14 function (supplemental Figure S2C,D).<sup>29,30,42</sup> Notably, PARP14 knockdown did not affect survival of BJAB cells (Figures 4A,C), despite these cells exhibit high PARP14 protein levels (Figure 3C) that were effectively knocked-down (Figure 4D), in accordance with their insensitivity to JNK2 depletion (see Figure 1F and 2A). Together, these data indicate that

knockdown of PARP14 resembles JNK2 knockdown, and suggest that JNK2 may mediate MM cell survival by regulating the levels of PARP14.

### **PARP14 mediates survival signaling by JNK2**

To test directly the role of PARP14 in JNK2-mediated survival in MM, we examined the effects of ectopic expression of PARP14 in JNK2-depleted cells using the lentiviral expression vector, pWPI.<sup>34</sup> Because the large size of the PARP14 ORF precluded efficient packaging of lentivirus particles, we generated PARP14( $\Delta$ N553), a truncated form of PARP14 that has previously been found to mimic its physiological functions (Figure 5A).<sup>29</sup> We tested whether apoptosis caused by JNK2 knockdown could be reversed by ectopic expression of PARP14( $\Delta$ N553). RPMI-8226 cells infected with either PARP14( $\Delta$ N553) or empty pWPI lentiviruses were double infected with shJNK2 lentivirus. PARP14( $\Delta$ N553)-overexpression was confirmed by WB (Figure 5B). Of note in PARP14( $\Delta$ N553)-overexpressing cells, levels of total PARP14 (endogenous plus exogenous) were comparable with those in control cells (Figure 5B; compare lane 1 with lane 3). As shown in Figure 5C (left panel), apoptosis induced by JNK2 knockdown was completely reversed by PARP14( $\Delta$ N553) overexpression in RPMI-8226 cells. Notably, the protective efficacy of PARP14( $\Delta$ N553) overexpression against this killing was comparable to that of control shNS-infected cells (see Figure 2A), whereas pWPI expression afforded no protection to apoptotic shJNK2-infected cells. Similar results were observed in additional MM cells (Figure 5C,D and data not shown). Remarkably, ectopic expression of PARP14( $\Delta$ N553) suppressed JNK1 activity in JNK2-depleted cells (Figure 5B; JNK1 K.A.). These results show that restoration of PARP14 expression rescues the apoptotic phenotype in myeloma cells caused by JNK2 depletion.

### **PARP14 binds and inhibits JNK1 to promote survival**

As a tool to further dissect the mechanism of JNK1 inhibition by JNK2 and PARP14, we co-expressed FLAG-tagged PARP14 in HEK293T cells with HA-JNK2p54, HA-JNK1p46 or HA-

empty vector, and investigated protein associations by combined immunoprecipitations and WB analyses. PARP14 specifically bound to JNK1p46, but not JNK2p54, and this binding was confirmed with PARP14( $\Delta$ N553) and PARP14( $\Delta$ N1470) (a mutant lacking the three macro domains) (Figure 6A; see also Figure 5A), indicating that the C-terminal of PARP14 is involved in this interaction. Importantly, an association between endogenous PARP14 and JNK1 was confirmed in both RPMI-8226 and BJAB cells. Anti-JNK1 antibodies co-immunoprecipitated PARP14 from parental cells, but not from cells depleted of PARP14 (Figure 6B). JNK1 was immunoprecipitated at comparable levels in both parental and shPARP14-infected cells (Figure 6B; WB: JNK1), and was not co-precipitated by isotype-matched control antibodies (data not shown). We conclude that endogenous PARP14 and JNK1 associate under physiological conditions.

To test whether the binding of PARP14 to JNK1 is sufficient to inhibit JNK1, we generated recombinant GST-tagged PARP14( $\Delta$ N1470) protein for use in JNK1 kinase assay. Neither recombinant PARP14( $\Delta$ N1470) nor control protein inhibited active JNK1 *in vitro* (supplemental Figures S3A,B), indicating that PARP14/JNK1 binding is not alone sufficient to explain the inhibitory effect of PARP14 on JNK1. Moreover, JNK1 still bound to MKK7, an upstream JNK activator, in HEK293T cells overexpressing PARP14 (supplemental Figure S3C).<sup>43</sup> Together, these results suggest that an additional inhibitory mechanism of JNK1 by PARP14 exists, *in vivo*.

We next investigated whether the protective function of PARP14 also depended on inhibition of JNK1. As shown in Figure 6C, co-silencing JNK1 with PARP14 completely rescued MM cells depleted of PARP14 from apoptosis, indicating that PARP14 suppresses JNK1-mediated apoptosis. The protective effect of JNK1 silencing was reminiscent to that seen in JNK2-depleted cells (Figure 2D-F), indicating that PARP14 and JNK2 have similar effects on JNK1-mediated apoptosis. Together, these findings identify PARP14 as the mechanistic link by which JNK2 suppresses JNK1, thus promoting myeloma cell survival.

**PARP14 inhibition can potentiate cytotoxicity of anti-myeloma agents**

As the JNK2-PARP14-JNK1 regulatory axis sustains the survival of MM cells, we investigated whether inhibition of PARP14 could be used as a therapeutic strategy in MM. First, we evaluated whether PARP14 expression had any impact on anti-MM therapy. Interrogation of the dataset GSE2658 revealed that high expression of PARP14 transcripts correlated with a significantly shorter survival in patients treated with the Total Therapy 3 protocol,<sup>39,40</sup> suggesting that PARP14 may be implicated in treatment outcomes (Figure 7A). Next, we assessed the effects of PJ-34 in combination with the anti-myeloma agent dexamethasone on MM cells. PJ-34 significantly enhanced cytotoxicity induced by dexamethasone (Figure 7B), suggesting an additive effect. To evaluate whether this effect of PJ-34 was attributable to PARP14 inhibition, we either silenced or overexpressed PARP14 in MM1.S cells and assayed their viability after treatment with dexamethasone. Depletion of PARP14 significantly enhanced the sensitization of MM1.S cells to dexamethasone compared with the control cells (Figure 7C). Conversely, MM1.S cells overexpressing truncated-PARP14 were resistant to dexamethasone-induced apoptosis compared with control vector. Moreover, addition of PJ-34 overcame this resistance and resulted in cell death (Figure 7D). Finally, PARP14 knockdown also sensitized MM1S cells to bortezomib, lenalidomide and, to a lesser extent, thalidomide other clinically relevant compounds used for the treatment of MM (Figure 7E,F).

**Discussion**

The identification of pathways that govern survival of myeloma plasma cells is of great interest for possible therapeutic applications, as deregulated survival is a key feature of myelomagenesis.<sup>1,2,4</sup>

In this study, we show that JNK2 is a master regulator of survival of myeloma cells, and define a new regulatory pathway linking JNK2 to JNK1 through regulation of a novel survival factor of B-lineage cells, PARP14.<sup>30</sup> Moreover, we report for the first time a correlation of PARP14 expression with tumor survival and progression in MM.

Elevated activity of p-JNK was found in myeloma plasma cells in all the BM trephine biopsies of newly diagnosed MM patients tested, but was nearly undetectable in normal plasma cells in the tonsillar samples. This basal JNK activity in myeloma plasma cells exhibits exclusive nuclear localization, which is consistent with published reports demonstrating the accumulation of p-JNK in the nucleus of cancer cells.<sup>18,23</sup> Unlike JNK1, JNK2 has autophosphorylation activity that leads to its constitutive activation in several tumour cell types.<sup>16,18,44</sup> Consistent with this, we found that the major JNK protein that is phosphorylated in MM cell lines and primary patient-derived cells is JNK2 (predominantly present as p-p54 isoform), but not JNK1 (predominantly present as p-p46 isoform), despite p46 and p54 JNK proteins being expressed at similar levels. Moreover, p-JNK2 levels and JNK2 activities in myeloma cells were higher than in normal B cells, whereas JNK1 activities were weak and remained unchanged, suggesting a pathogenic role for JNK2 in this disease.

We systematically evaluated the functional significance of constitutive JNK2 activity using shRNA interference, and demonstrated that JNK2 is essential for survival of myeloma cells. In this system, silencing of JNK2 or JNK1 expression was found to be highly specific, as levels of ERK- and p38-MAPK were not affected. The survival function of JNK2 is not shared by JNK1. Indeed, while depletion of endogenous JNK2 protein enhanced apoptosis in MM cell lines, reduction in the protein levels of JNK1 did not affect the growth rates of these cancerous cells. Moreover, constitutive activation of JNK2 negatively regulated activity of JNK1 since increased JNK1 activity was observed in JNK2-depleted cells. Oncogenic function of JNK2 on survival was depending on JNK1-mediated apoptosis. Our findings demonstrate that co-silencing of

JNK1 with JNK2 completely rescued MM cells from apoptosis induced by JNK2 depletion. This was further supported by our observations that JNK1 co-silencing inhibited activation of effector caspase-3 triggered by silencing of JNK2 in myeloma cell lines. The fact that no alteration of phosphorylation of ERK and p38-MAPK was observed in JNK2-depleted cells further suggested that JNK1 is the specific MAPK involved in shJNK2 inducing cell apoptosis.

Our results indicate that JNK2 is not a universal oncogene, as knockdown of JNK2 expression in the BL cell line BJAB did not alter the basal cell viability. Recent data has implied that JNK2-mediated cell survival involves the regulation of selective pro-survival proteins or tumor suppressors in specific cellular contexts. JNK2 cooperates with Ras to promote epidermal neoplasia,<sup>45</sup> phosphorylates oncoproteins such as STAT3 in non-small cell lung carcinoma and Akt in glioblastoma,<sup>16,18</sup> and contributes to the inhibition of tumour suppressors in breast and colon cancer cells.<sup>46,47</sup> Interestingly, we found that JNK2 depletion leads to a reduction of PARP14 levels in MM cells, but not in BJAB, suggesting that the JNK2-dependent regulation of PARP14 is limited to terminally differentiated B-cells. Elevated expression levels of PARP14 were found in MM cell lines as well as primary CD138+ MM cells and, intriguingly, correlated with the nuclear activity of JNK2 in MM primary tumors. PARP14 levels also appear to have important clinical implications for patients with MM as PARP14 gene expression increases with disease progression from normal BM plasma cells to early (MGUS) and then to active stages of MM. Furthermore, knockdown of PARP14 recapitulated knockdown of JNK2 in MM cells by mediating apoptosis, consistent with the hypothesis that JNK2 mediates survival by regulating PARP14 expression. Complementary experiments further supported this conclusion by showing that the increased apoptosis in JNK2-depleted cells was completely suppressed by expression of PARP14. Thus, the function of JNK2 to inhibit apoptosis can be replaced by PARP14, consistent with the established role of this protein as a protective factor in primary murine B-lymphocytes.<sup>30</sup> The obvious question that remains to be addressed relates to the mechanisms of reduced PARP14 expression caused by JNK2 depletion. JNK proteins

regulate the activity of several transcription factors, such as c-Jun and ATF-2, and the stability of non-transcription factors, such as Bcl2.<sup>11</sup> Therefore, it is possible that JNK2 may indirectly regulate PARP14 expression at either transcriptional or posttranscriptional levels. Further studies are required to address this question.

What is the molecular mechanism by which JNK2 and PARP14 proteins cooperate in regulating MM cell survival? The JNK2-dependent suppression of JNK1 is apparently mediated through PARP14, because JNK1 activity is similarly increased in PARP14-depleted cells and the suppression of JNK1 was restored by overexpression of PARP14( $\Delta$ N553) in cells depleted of JNK2. Consistent with this, apoptosis caused by PARP14 knockdown in MM cells was completely reversed by JNK1 co-silencing, indicating JNK1 as a direct mediator of apoptosis triggered by PARP14 knockdown.

Along with the three tandem “macro” domains, PARP14 contains a WWE domain and a catalytic PARP domain at the C-terminal.<sup>38,48</sup> The WWE domain is predicted to mediate protein-protein interactions in ubiquitination and ADP-ribose conjugation systems.<sup>49</sup> Indeed, it has been shown that PARP14 associates with Stat6 and phosphoglucose isomerase through its C-terminus.<sup>29,49</sup> Furthermore, PARP14 activity was shown to be essential in mediating IL-4-induced protection of murine B-lymphocytes against apoptosis.<sup>30</sup> Although we have not addressed whether the enzymatic activity of PARP14 is strictly required for the inactivation of JNK1 kinase, we found that PARP14 specifically interacts with JNK1 through its C-terminal portion and that overexpression of the latter completely suppressed JNK1 activity. We also found that blocking PARP activity using PJ-34 resulted in JNK1 activation and apoptosis. Accordingly, neither PARP14/JNK1 interaction was alone sufficient for JNK1 suppression nor prevented JNK1 from binding to MKK7, an upstream JNK1 activator. Therefore, it is possible that the PARP14 catalytic activity might be involved in JNK1 inactivation. Studies to define the role of the PARP14 activity in inhibiting JNK1 kinase activity in more detail are under way.

Finally, we observed that high PARP14 mRNA expression correlated with poor survival outcomes in newly diagnosed MM patients treated with total therapy 3 protocol.<sup>39,40</sup> In line with these clinical observations, we showed that inhibition of PARP14 can cooperate with anti-myeloma agents in inducing more effective cell death, suggesting that PARP14 could be targeted to improve anti-MM therapies. Therefore, it will be of interest to examine the role of PARP14 in development of drug resistance.

In summary, we identify a novel pathway whereby constitutively active JNK2 promotes survival via PARP14, which in turn associates with JNK1 and inhibits its kinase activity; this inhibition is crucial for the suppression of basal apoptosis in myeloma cells (Figure 7G). Our findings define for the first time the individual role of JNK1 and JNK2 in MM and demonstrate that PARP14 is a novel physiological downstream effector of JNK2, thus expanding the number of specific targets downstream of JNK2. These results may open new avenues for therapeutic intervention in MM.

## **Material and Methods**

### **Patient samples**

After approval from the Hammersmith Hospitals NHS Trust Research Ethics Committee and informed consent according to the Declaration of Helsinki, BM samples were obtained from patients with MM undergoing routine diagnostic procedure.

Mononuclear cells were isolated from BM aspirates by Ficoll gradient centrifugation, and primary CD138+ MM cells were positively sorted using CD138-conjugated magnetic beads (Miltenyi-Biotec, Surrey, UK) as previously reported.<sup>32</sup>

**Cells and reagents**

Human MM cell lines (RPMI-8226, JJN-3, MM1.S, OPM-2, KMS12-BM, KMS11 and U266) and Burkitt's lymphoma lines (BJAB, Ca46, and Raji) were cultured in RPMI1640 (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (Sigma, Gillingham-Dorset, UK), 100 U/mL penicillin, 100mg/mL streptomycin, and 2mM L-glutamine (Invitrogen) in a 5%CO<sub>2</sub> incubator at 37°C.<sup>32,51</sup> D-MEM (Invitrogen) was used as culture medium to grow human HEK293T cells. Normal B cells were isolated from peripheral blood of healthy donors using the B-cell isolation kit II (Miltenyi-Biotec), according to the manufacturer's instructions. Dexamethasone and PJ-34 were obtained from Sigma. Bortezomib was obtained from the pharmacy of Hammersmith Hospital. Thalidomide and Lenalidomide were obtained from Tocris Bioscience (Bristol, UK) and Selleck Chemicals (Huston, USA) respectively.

**Plasmids, co-transfection, protein purification, lentiviral preparations and infections**

pcDNA-FLAG, pSR $\alpha$ -HA-JNK1, pcDNA-HA-JNK2 were used previously.<sup>52</sup> pcDNA-FLAG-PARP14 was obtained by sub-cloning the full-length human cDNA of PARP14 (provided by Dr Michael Johns) between the KpnI-XhoI restriction site of pcDNA-FLAG. pcDNA-FLAG-PARP14( $\Delta$ N553) and pcDNA-FLAG-PARP14( $\Delta$ N1470), encoding amino acids 553-1801 and 1470-1801 of human PARP14, respectively, were obtained by linearizing pcDNA-PARP14(FL) with appropriate restriction enzymes and replacing original fragments with oligonucleotide linkers. For co-immunoprecipitations, HEK293T cells were co-transfected using the calcium phosphate method with HA-tagged and FLAG-tagged plasmids, as described before.<sup>52</sup>

To generate pGEX-PARP14( $\Delta$ N1470), the cDNA PmeI-PstI fragment of pcDNA-FLAG-PARP14( $\Delta$ N1470) was insert into the BamHI-SmaI sites of pGEX1 along with an appropriate oligonucleotide linker. GST-PARP14( $\Delta$ N1470) were expressed in *E.coli* and purified as previously described.<sup>52</sup>

Oligonucleotides to obtain shRNA constructs were cloned into the lentiviral vector, pLL3.7, using the HpaI-XhoI restriction sites.<sup>33,34</sup> Oligonucleotides used are listed in Supplemental Table S1. Non-specific shRNA (shNS) was previously described.<sup>33</sup> We also used a lentiviral vector pWPI for complementary experiments.<sup>34</sup> The expression constructs pWPI-FLAG-PARP14, pWPI-FLAG-PARP14( $\Delta$ N553) and pWPI-HA-JNK2 were generated by subcloning the full-length PARP14, PARP14( $\Delta$ N553) or JNK2 cDNA sequences into the PmeI restriction site of pWPI. All constructs were confirmed by nucleotide sequencing. Lentiviral preparations were obtained, as previously described.<sup>33</sup> Supernatants were harvested, concentrated and then used to infect MM cell lines in presence of 8 $\mu$ g/ml polybrene (Sigma)

### **Apoptosis and cell viability assays**

Apoptosis was assessed by FCM analysis using PI nuclear staining, as previously described,<sup>33,51</sup> and Annexin-V-eFluor450 and 7-amino-actinomycin D (7AAD) (eBioscience, Hatfield, UK) staining, according to the manufacturer's instructions. The percentage of apoptotic cells was calculated on the total population, gating out the cellular fragments using the FSC/SSC parameter. Cell viability was assessed using MTT reagent according to the manufacturer's protocol (Sigma). Proportion of live GFP+ was evaluated over time by FCM analysis, and expressed relative to the proportion at 2 or 4 days after lentiviral infection.

### **Western blot, kinase assay, co-immunoprecipitation and antibodies.**

WB, co-immunoprecipitation, JNK1 and JNK2 kinase assays (K.A.) were performed as described previously.<sup>33,51,53</sup> Antibodies used in these protocols included JNK1 and JNK1/2 (BD Biosciences, Heidelberg, Germany); caspase-3, caspase-9, phospho-JNK, phospho-p38, phospho-ERK1/2, JNK2, p38, ERK1/2 (Cell Signaling, Danvers, MA, USA); anti-HA-probe, PARP9,  $\beta$ -actin (Santa Cruz, Heidelberg, Germany); PARP1 (Calbiochem, Lutterworth, UK); PARP14 and anti-FLAG-probe (Sigma).

### **Immunohistochemistry**

Sample preparation was performed as previously described.<sup>54</sup> Rabbit anti-human PARP14 and phospho-JNK were used in a dilution of 1:50. The staining in the myeloma samples was reviewed and scored by a qualified hematopathologist (K.N.) in a blinded fashion. Stained sections were analyzed with a light microscope (BX51, Olympus) carrying a DP70 digital camera.

### **Gene-expression profiling**

Publicly available gene-expression data for a series of MM (GSE2658 and GSE5900) and Burkitt's lymphoma (GSE12453) primary samples profiled on Affymetrix U133 (Affymetrix, Santa Clara, CA, USA) platforms were downloaded and analyzed against human PARP14 (probe 224701\_at).<sup>39-41</sup>

### **Statistical analysis**

Data are shown as mean  $\pm$  SD of three experiments. Statistical significances of differences were determined using appropriate tests as described in figure legends. The minimal level of significance was  $P < 0.05$ .

### **Conflict of Interest**

The authors declare no conflicts of interest.

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## Figure Legends

**Figure 1. JNK2 is constitutively activated in MM primary tumors and cell lines and targeting JNK2 results in MM cell death (A)** Immunohistochemical analysis of phospho-JNK (p-JNK) expression in BM biopsies of newly diagnosed MM patients (Pt=4) and normal

tonsillar samples (n=3). Representative sections are shown. Positive staining for p-JNK is demonstrated in myeloma plasma cells (white arrowheads) by brown nuclear signal. Positivity for p-JNK is completely absent in normal plasma cells (black arrowheads) in the tonsillar sample. Images are showing x600 magnification. **(B)** Western blots (left) with antibodies against phosphorylated or total JNK1 and JNK2 showing increased expression of p-JNK in CD138-purified myeloma from 6 additional newly diagnosed patients with MM (Pt. 5-10) compared with normal B cells isolated from human PBMCs of three healthy donors. Right, densitometry analysis of p-p54 and p-p46 bands versus total JNK2p54 and JNK1p46, respectively. Intensity of p-p54 (p-JNK2) is significantly higher than p-p46 (p-JNK1). The two-tailed Wilcoxon signed-rank test was used to compare expression of p-p54 and p-p46 in CD138-purified myeloma cells from MM patients. Each symbol represents an individual patient; horizontal lines indicate the median. **(C)** Western blots showing increased expression of p-JNK in a collection of human MM cell lines compared with normal B cells, and increased levels of p-p54 (p-JNK2) compared to p-p46 (p-JNK1). In vitro kinase assays (K.A.) with GST-c-Jun as substrate showing JNK1 and JNK2 activities in indicated MM cell lines and normal B cells. Total JNK1 and JNK2, and  $\beta$ -actin were used as loading control. **(D)** Western blots with antibodies against both spliced variants of JNK1 (JNK1p46, JNK1p54) and JNK2 (JNK2p46 and JNK2p54) showing the knockdown of either JNK1 or JNK2 proteins in RPMI-8226 cells infected with lentiviruses expressing JNK1, JNK2, but not control (lane NS), shRNA. Total p38, ERK1/2 and  $\beta$ -actin were used as loading control. **(E, F)** Analysis of GFP expression in RPMI-8226 MM and BJAB Burkitt's lymphoma cells infected as in (D) showing a reduction of GFP-positive cells over time in RPMI-8226 shJNK2 cells, but not in shJNK1, control or BJAB cells. Proportion of GFP-positive cells were assessed at indicated times and expressed relative to the proportion on day 2 after infection. Values are the mean  $\pm$  SD of three independent cultures. **(G)** JNK1 and JNK2 levels were assessed by Western blot in BJAB cells infected as in (F).

**Figure 2. JNK2 promotes MM cell survival by suppressing JNK1-mediated apoptosis.**

(A) RPMI-8226 and MM1.S MM cell lines and BJAB cells were infected with lentiviruses expressing JNK1, JNK2 or control non-specific (NS) shRNA, and after the times indicated, stained with propidium iodide and analyzed by FCM. Shown is the percentage of apoptotic cells in the population (sub-G1 DNA content). Values are the means  $\pm$  SD of three independent experiments. (B) Human myeloma JJN3 cells were infected as described in (A), and six days after infection the percentage of AnnexinV<sup>+</sup> cells showing either AnnexinV<sup>+</sup>/7AAD<sup>-</sup> (early apoptosis) and AnnexinV<sup>+</sup>/7AAD<sup>+</sup> (late apoptosis) was assayed by FCM using AnnexinV/7AAD staining. Data are expressed as mean  $\pm$  SD of three separate experiments. (C) Western blots, in-vitro JNK1 and JNK2 kinase assay (K.A) of lysates from RPMI-8226 and JJN3 cells infected as in (A) for 8 or 10 days, respectively, showing cleavage of caspases and PARP1 as well as activation of JNK1 and JNK2 using the indicated antibodies and GST-c-Jun as substrate, respectively. Closed and open arrowheads indicate the pro-cleaved and cleaved (active) products of the indicated proteins, respectively. Activation of p38 and ERK1/2 were analyzed by immunoblotting with the indicated phospho-specific (p) antibodies.  $\beta$ -actin was used as loading control. (D-E) Co-silencing of JNK1 and JNK2 rescues MM cells from apoptosis induced by JNK2 depletion. RPMI-8226, MM1.S and JJN3 cells were infected with lentiviruses expressing JNK2 shRNA or JNK1/JNK2 shRNA (ie silencing simultaneously both proteins), and the percentage of sub-G1 population and AnnexinV<sup>+</sup> cells were determined by FCM at day 6, 10 or 8 of infection. (F) Western blots of extracts from MM cells used in (D-E) showing levels of cleaved-active caspase-3 (Cl. caspase-3), and total JNK1/JNK2.  $\beta$ -actin was used as loading control. Representative results from at least three experiments are shown.

**Figure 3. PARP14 is overexpressed in MM cells, and its expression correlates with disease progression and depends on JNK2.**

(A) Western blots showing a reduction of PARP14 levels in MM cells (RPMI-8226, MM1.S and JJN3) but not in BJAB cells infected with

lentiviruses expressing JNK2 shRNA compared to JNK1 shRNA or control NS shRNA. PARP9 and  $\beta$ -actin levels were analyzed with appropriate antibodies as controls. **(B)** Immunohistochemical analysis of PARP14 expression in BM biopsies of newly diagnosed MM patients (Pt=4) and normal tonsillar samples (n=3). Representative sections are shown. Positive staining for PARP14 is demonstrated in myeloma plasma cells (white arrowheads) by brown nuclear signal. Positivity for PARP14 is completely absent in plasma cells (black arrowheads) in the tonsillar sample. Images are showing x600 magnification. **(C)** Western blots showing PARP14 expression in CD138<sup>+</sup>-selected cells from the BM aspirates of 6 additional MM patients (Pt. 5-10) and B cells from PBMCs of three healthy donors as well as indicated MM and Burkitt's lymphoma cell lines.  $\beta$ -actin was used as loading control. **(D, left)** Gene-expression analysis of PARP14 from oligonucleotide microarray data GSE2658 and GSE5900 (Ref. 39,40) showing significantly higher expression of PARP14 transcripts ( $\log_2$  transformed, median-centered values) in MM plasma cells (n=414) compared to normal BM (n=22) or monoclonal gammopathy of unknown significance (MGUS) (n=44) plasma cells ( $P < 0.0001$ , nonparametric Kruskal-Wallis one-way analysis of variance; \* $P < 0.05$  and \*\*\* $P < 0.001$ , Dunn's multiple comparison post-hoc tests). For each box plot, the whiskers represent the 2.5-97.5th percentile range of values, the lower and up boundaries denote the 25th and the 75th percentile of each dataset, respectively, and the horizontal line represents the median value for each group. **(D, right)** Gene-expression profiling of PARP14 from oligonucleotide microarray data GSE12453 (Ref. 41) showing no significant differences of PARP14 transcripts levels between normal purified tonsillar germinal center (GC) B cells (centrocytes and centroblasts) (n=5) and Burkitt's lymphoma (BL) cases (n=5) ( $P = 0.1013$ , nonparametric Kruskal-Wallis one-way analysis of variance). The horizontal line indicates the mean.

**Figure 4. PARP14 expression is required for survival of MM cells.** **(A)** RPMI-8226, MM1.S and BJAB cells were infected with lentiviruses expressing PARP14 or control NS shRNA, and

after the times indicated, stained with PI and analyzed by FCM. Shown is the percentage of apoptotic cells in the population (sub-G1 DNA content). Values are the means  $\pm$  SD of three independent experiments. **(B)** JJN3 cells were infected as described in (A), and the percentage of AnnexinV<sup>+</sup> cells showing either AnnexinV<sup>+</sup>/7AAD<sup>-</sup> (early apoptosis) and AnnexinV<sup>+</sup>/7AAD<sup>+</sup> (late apoptosis) was assayed by FCM using AnnexinV/7AAD staining. Data are expressed as mean  $\pm$  SD of three separate experiments. **(C)** Western blots and in-vitro JNK1 kinase assay (K.A) of lysates from RPMI-8226, JJN3 and BJAB cells infected as in (A-B) for 10 days, showing cleavage of indicated caspases and PARP1 as well as activation of JNK1 using the indicated antibodies and GST-c-Jun as substrate, respectively. Closed and open arrowheads indicate the pro-cleaved and cleaved (active) products of the indicated proteins, respectively.  $\beta$ -actin was used as loading control **(D)** Western blots showing the knockdown of PARP14 levels in indicated MM cell lines and BJAB cells infected with lentiviruses expressing PARP14, but not control (lane NS), shRNA. PARP9 and  $\beta$ -actin were used as loading control. **(E)** Analysis of GFP expression in OPM2 and KMS12-BM infected as in (D) showing a depletion of GFP-positive cells over time in shPARP14 cells, but not in control cells. Proportion of GFP-positive cells were assessed at indicated times and expressed relative to the proportion on day 4 after infection. A representative experiment of three is shown.

**Figure 5. PARP14 suppresses JNK1-mediated apoptosis in JNK2-depleted cells (A)**

Schematic representation of full-length (FL) (amino-acid residues 1 to 1801) FLAG-tagged PARP14 [FLAG-PARP14 (FL)] and the C-terminal fragments of PARP14, FLAG-PARP14( $\Delta$ N553) and FLAG-PARP14( $\Delta$ N1470), used in complementary and co-immunoprecipitation experiments. The three macro-domains (Macro), the PARP-domain and the protein-protein interaction domain WWE are indicated. **(B)** Representative western blots showing ectopic expression of FLAG-PARP14( $\Delta$ N553) in RPMI-8226 cells infected with lentiviruses expressing FLAG-PARP14( $\Delta$ N553) or empty virus (pWPI), and then double

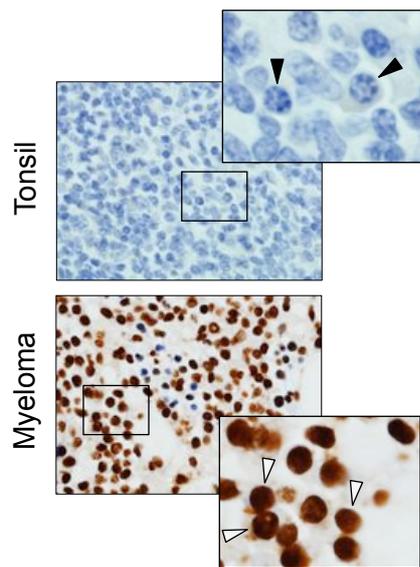
infected with lentiviruses expressing JNK2 shRNA. Endogenous [FL] and exogenous PARP14 [FLAG-PARP14( $\Delta$ N553)] are indicated. Kinase assays (K.A.) showing inhibition of JNK1 activity in the same RPMI-8226 infected cells. GST-c-Jun was used as substrate. Total JNK1/2 and  $\beta$ -actin was used as loading control. **(C-D)** Ectopically expressed FLAG-PARP14( $\Delta$ N553) rescues JNK2-depleted cells from apoptosis. Myeloma cell lines were infected as in (B), and apoptosis was assessed by FCM using PI (C) or AnnexinV/7AAD staining (D). Shown are bar graphs representing the mean ( $\pm$  SD) percentage of apoptotic cells in the population (sub-G1 DNA content or AnnexinV+ cells) in three independent experiments.

**Figure 6. PARP14 specifically interacts with JNK1.** **(A)** HEK293T cells were transfected with the indicated FLAG-tagged PARP14 and HA-tagged empty vector (HA-vector), HA-JNK1 or HA-JNK2. After 24 hours, cells were harvested and lysed. Lysates were immunoprecipitated (IP) with anti-FLAG and subject to western blots (WB) as indicated. Results are representative of three experiments. **(B)** Western blots with anti-JNK1 immunoprecipitates (top) or lysates (bottom) from RPMI-8226 (left) and BJAB (right) shPARP14 or uninfected (parental) cells, showing association of endogenous PARP14 with JNK1. **(C)** Co-silencing of JNK1 with PARP14 rescues MM cells from apoptosis induced by shPARP14. RPMI-8226, MM1.S and JJN3 cells were infected with lentiviruses expressing JNK1 or control NS shRNA, double infected with shPARP14, and then the percentage of AnnexinV+ cells showing either AnnexinV+/7AAD- (early apoptosis) and AnnexinV+/7AAD+ (late apoptosis) was assayed by FCM using AnnexinV/7AAD staining. Values are the mean  $\pm$  SD of three independent experiments.

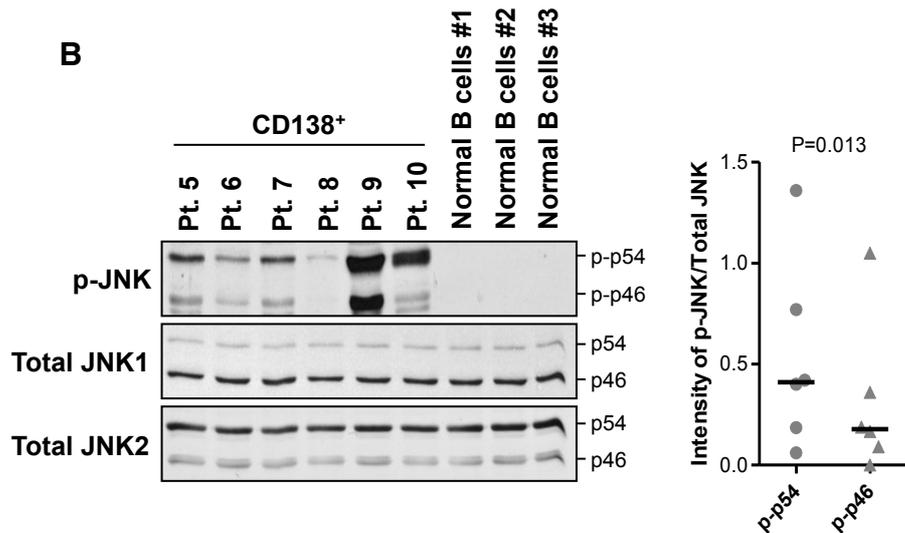
**Figure 7. PARP14 inhibition enhances cytotoxicity of anti-myeloma agents.** **(A)** High PARP14 expression significantly correlates with poor survival outcomes post therapy. Shown are the Kaplan-Meier overall survival (OS) curves of MM patients treated with total therapy 3

(TT3) according to PARP14 expression above or below the median value of 1082 based on a dataset GSE2658 (Ref. 39,40) ( $P= 0.0202$ ). **(B)** RPMI-8226 and MM1.S viability, as assessed by MTT assay, 48 hours after treatment with dexamethasone (Dex) [5 $\mu$ M or 10 $\mu$ M], PJ-34 [50 $\mu$ M], or both in combination, is shown. Values are the mean  $\pm$  SD of triplicate cultures.  $**P<0.01$  and  $***P<0.001$ , two-tailed  $t$  tests. **(C)** MM1.S were infected with the indicated lentiviruses and treated with dexamethasone [10 $\mu$ M]. Cell viability was measured by MTT assay at 48 hours after treatment. Values are the mean  $\pm$  SD of triplicate cultures.  $**P<0.01$  and  $***P<0.001$ , two-way ANOVA with Bonferroni's post-hoc tests. **(D)** MM1.S infected with lentiviruses expressing FLAG-PARP14( $\Delta$ N553) or empty virus (pWPI) were cultured for 48 hours with dexamethasone [5 $\mu$ M or 10 $\mu$ M] alone or in combination with PJ-34 [50 $\mu$ M]. The bar graphs show the percentage of apoptotic cells in the population (sub-G1 DNA content) analyzed by FCM. Values are the mean  $\pm$  SD of triplicate cultures.  $***P<0.001$ , two-way ANOVA with Bonferroni's post-hoc tests. **(E)** MM1.S were infected as in (C) and then treated with bortezomib (Bortez) [5nM]. Cell viability was measured by MTT assay at 24 hours after treatment. Values are the mean  $\pm$  SD of triplicate cultures.  $***P<0.001$ , two-way ANOVA with Bonferroni's post-hoc tests. **(F)** MM1.S were infected as in (C) and then treated with thalidomide (Thal) [25 $\mu$ g/ml], lenalidomide (Len) [1 $\mu$ M], or left untreated for 72 hours. Cell viability was measured by MTT assay. Values are the mean  $\pm$  SD of triplicate cultures.  $***P<0.001$ , two-way ANOVA with Bonferroni's post-hoc tests. **(H)** Schematic model for JNK2-dependent myeloma cell survival. JNK2 suppresses basal apoptosis in myeloma cells via PARP14. Constitutive JNK2 activity regulates levels of PARP14, which in turn inhibits JNK1, thereby suppressing the activation of endogenous apoptotic pathways.

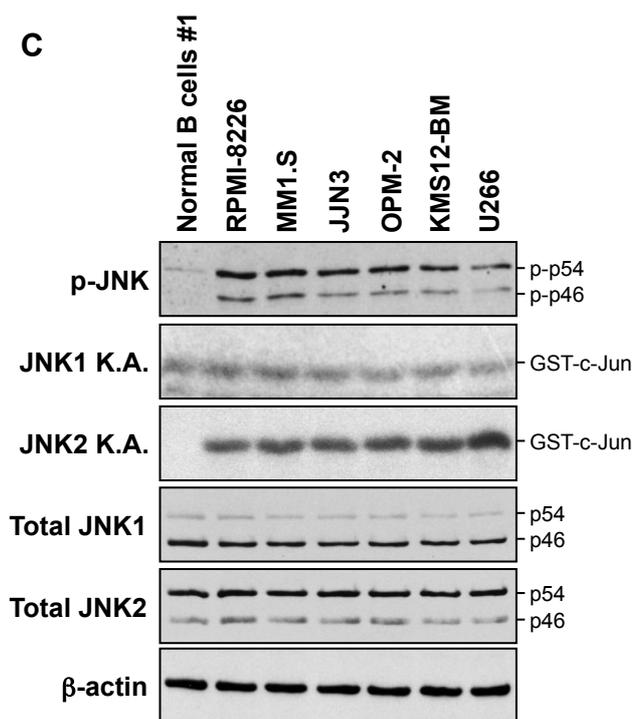
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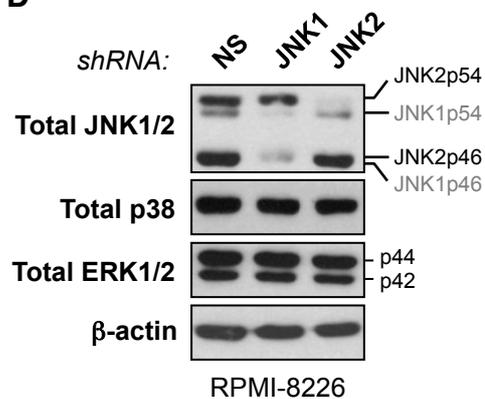
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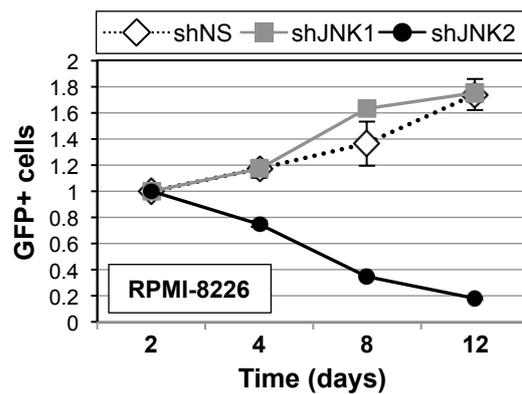
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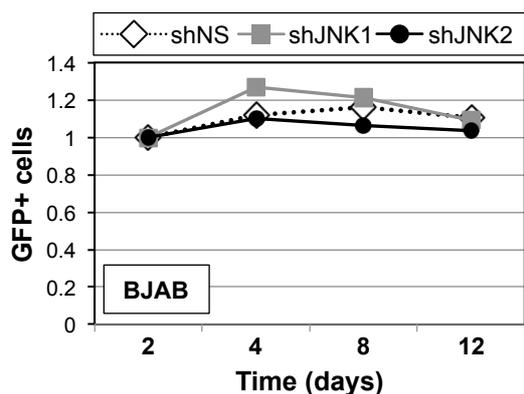
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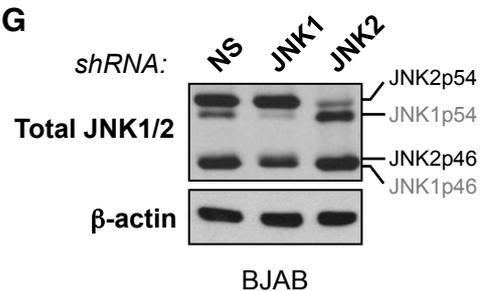
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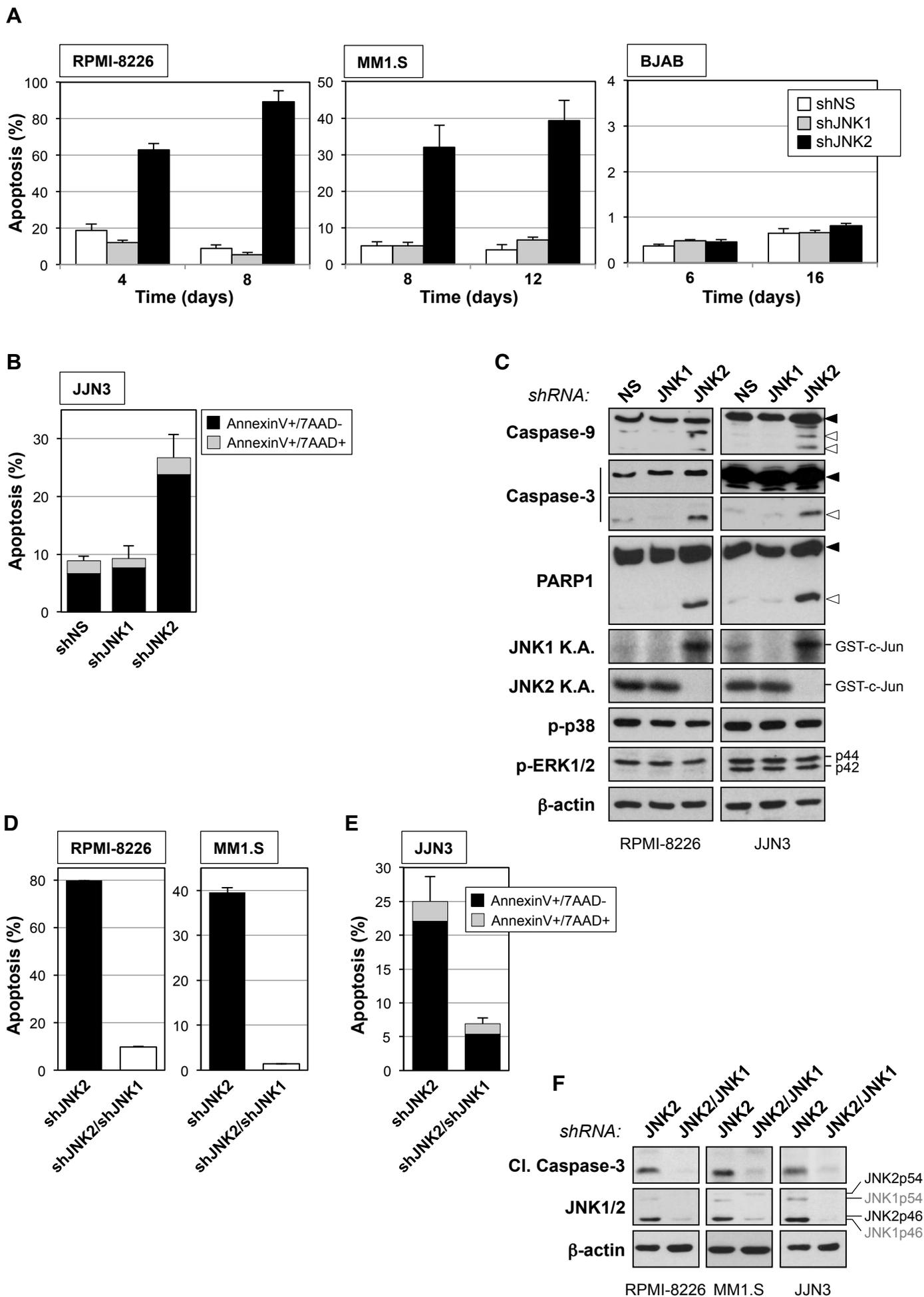


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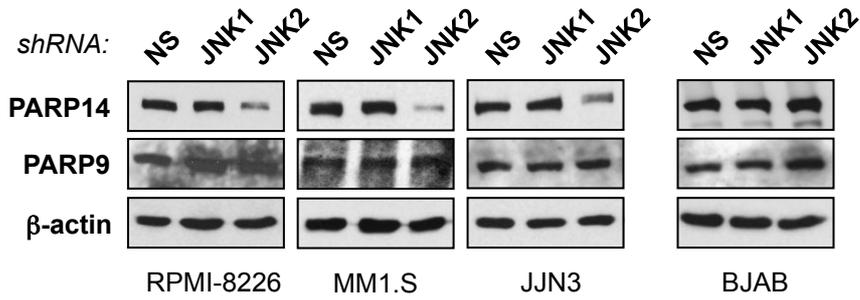


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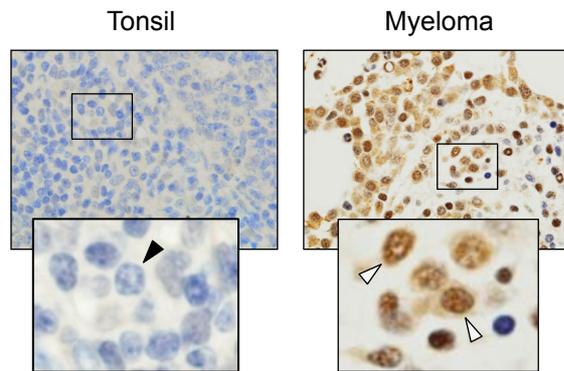




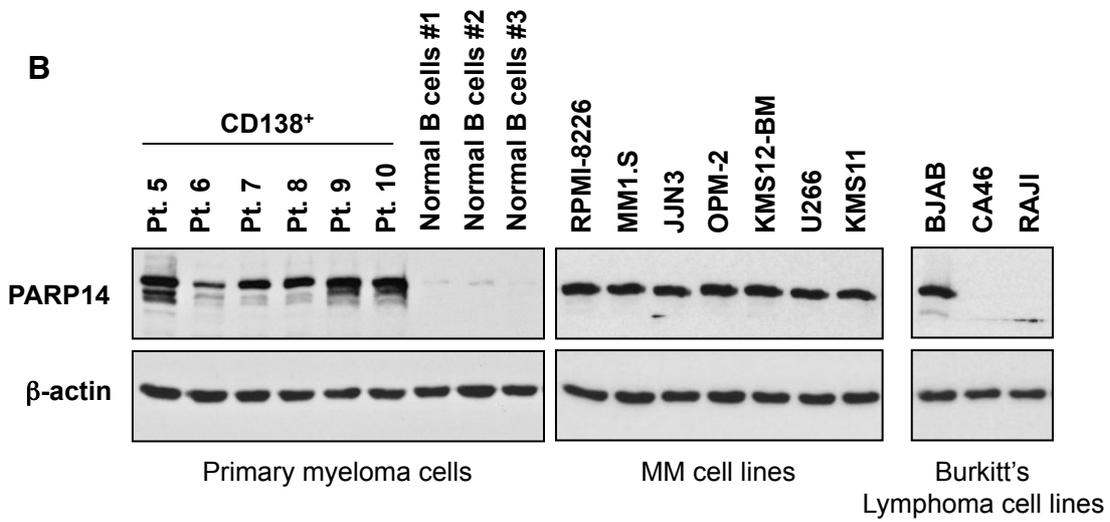
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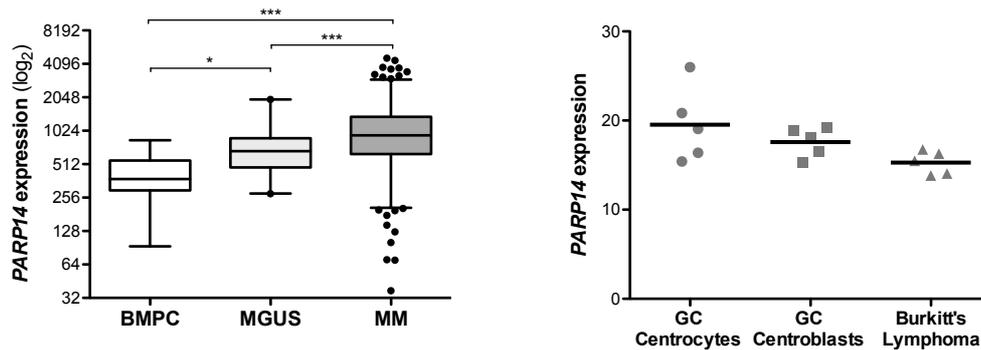
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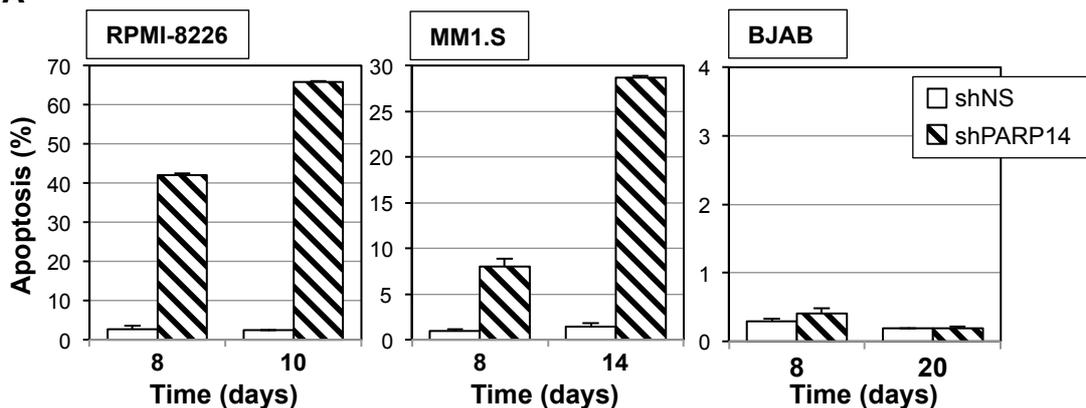
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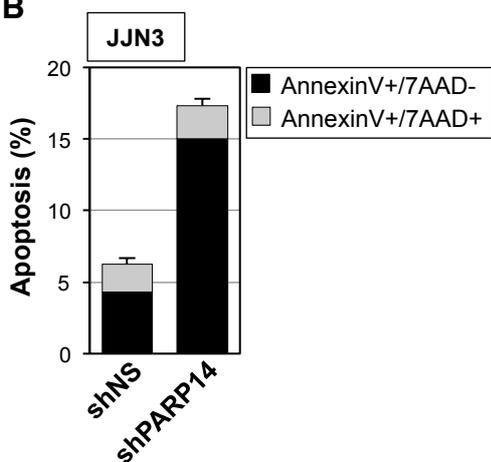
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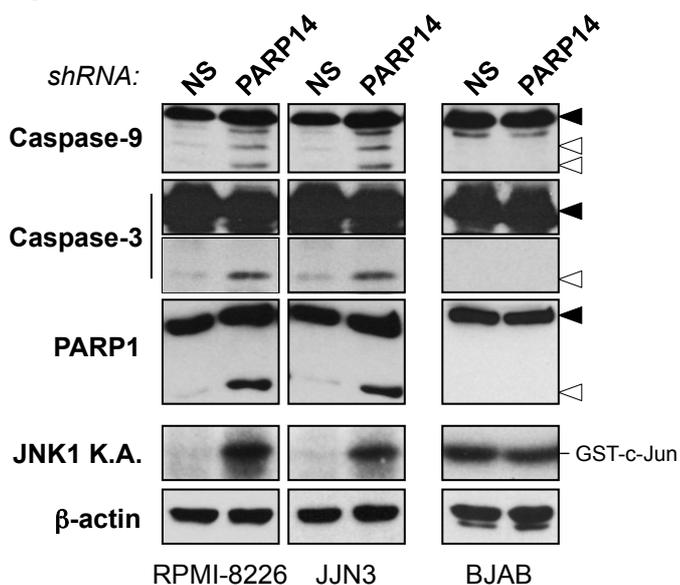
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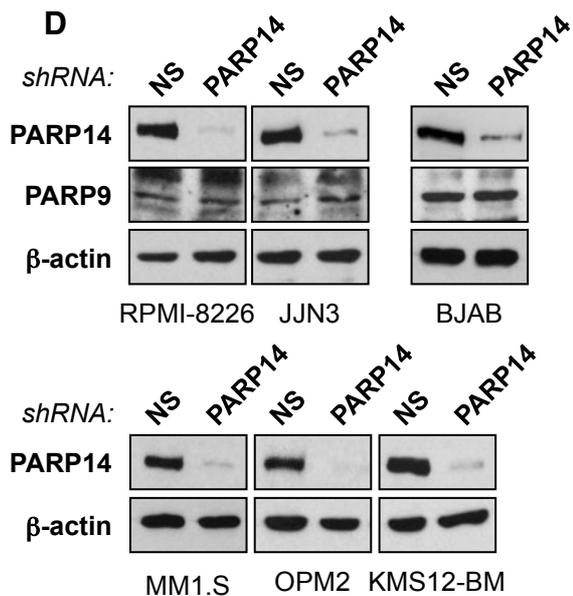
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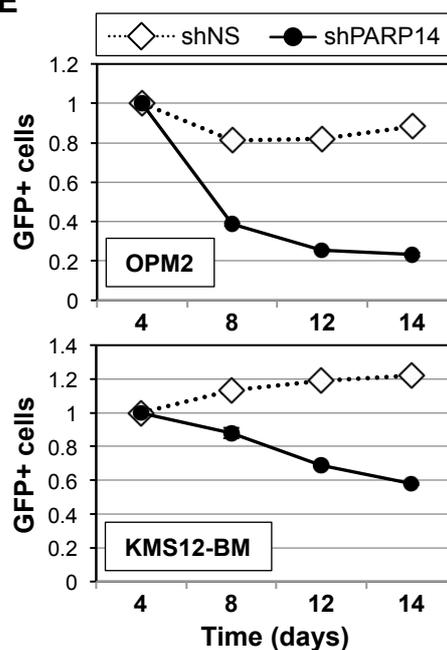
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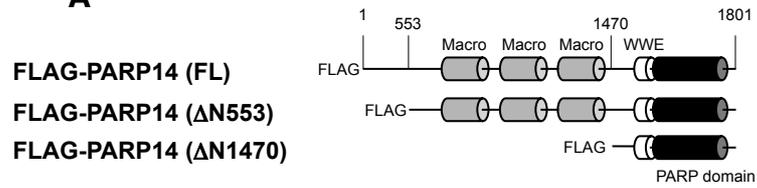
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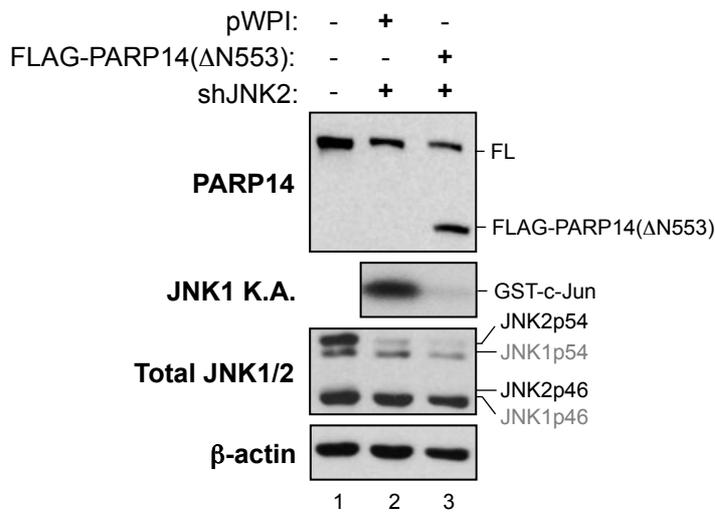
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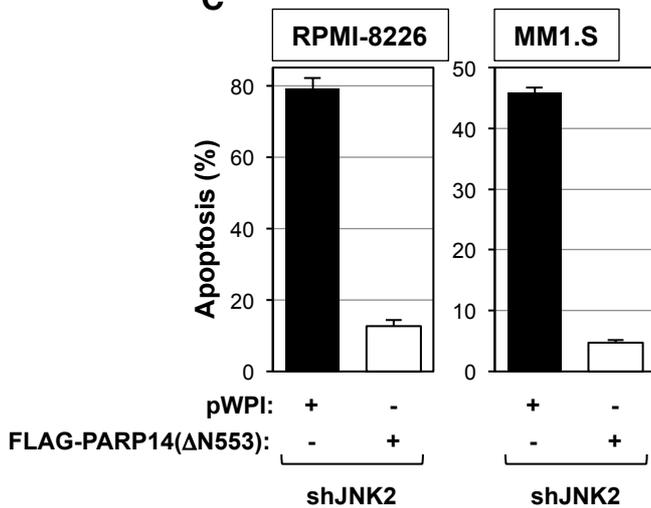
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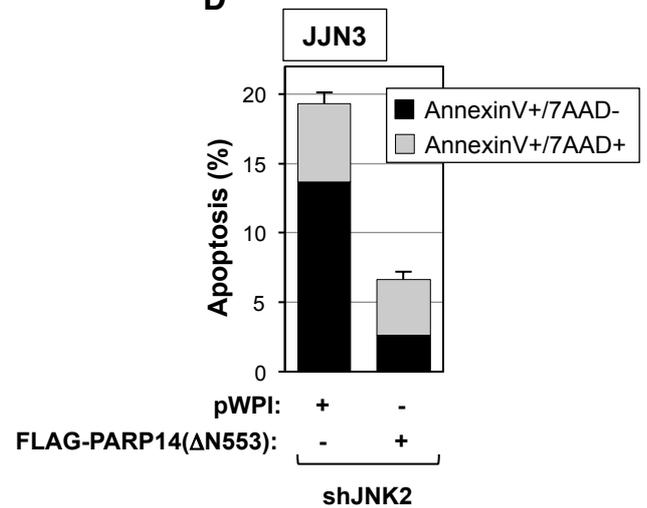
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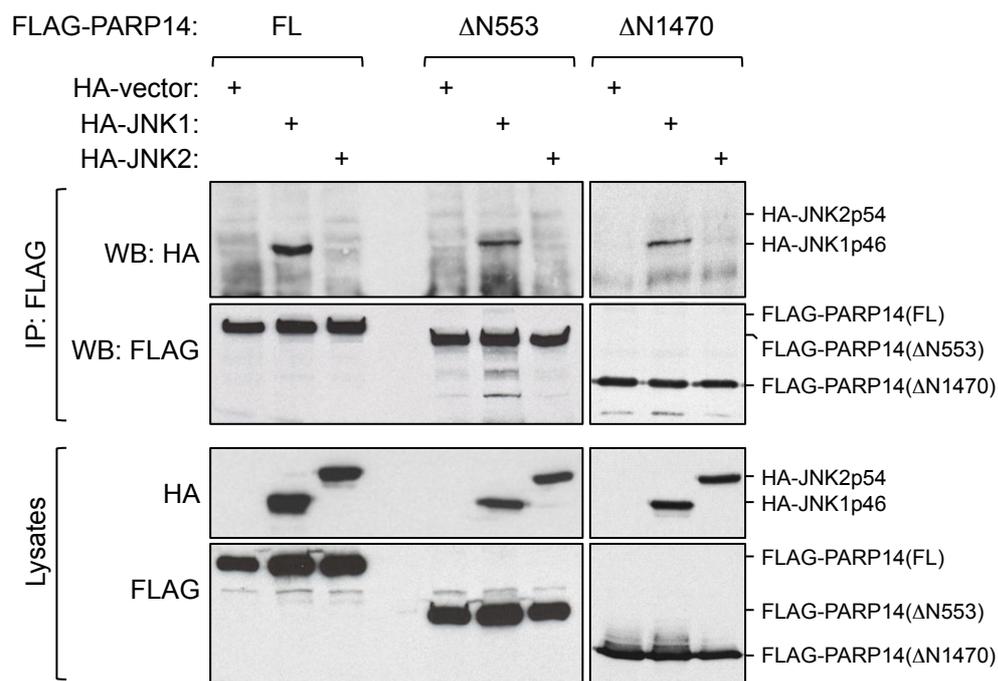
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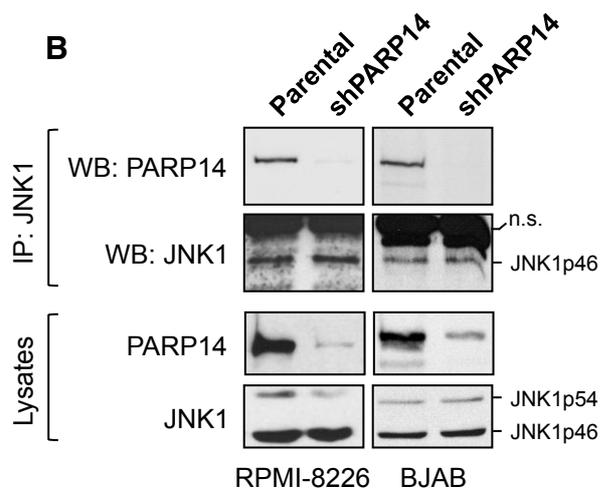
**D**



**A**



**B**



**C**

