

1 *Theileria* highjacks JNK2 into a complex with the macroschizont 2 GPI-anchored surface protein p104

3 **Perle Latré De Laté^{1,2*}, Malak Haidar^{1,2,3*}, Hifzur Ansari³, Shahin Tajeri^{1,2}, Eszter**
4 **Szarka⁴, Anita Alexa⁴, Kerry Woods⁵, Attila Reményi⁴, Arnab Pain^{3,6}, Gordon**
5 **Langsley^{1,2,#}**

6 ¹Laboratoire de Biologie Cellulaire Comparative des Apicomplexes, Faculté de Médecine,
7 Université Paris Descartes - Sorbonne Paris Cité, France.

8 ²Inserm U1016, Cnrs UMR8104, Cochin Institute, Paris, 75014 France.

9 ³Pathogen Genomics Laboratory, Computational Bioscience Research Center, King Abdullah
10 University of Science and Technology (KAUST), Thuwal-23955-6900, Kingdom of Saudi
11 Arabia.

12 ⁴Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of
13 Sciences, Budapest, Hungary

14 ⁵University of Bern Vetsuisse Faculty, Switzerland

15 ⁶Global Station for Zoonosis Control, Global Institution for Collaborative Research and
16 Education (GI-CoRE), Hokkaido University, N20 W10 Kita-ku, Sapporo, Japan.

17 *Co first authors with equal contribution.

18 #Corresponding author: Gordon Langsley; Email: gordon.langsley@inserm.fr

Abstract:

19 *Theileria* is a unique apicomplexan parasite capable of transforming its host cell into a
20 disseminating tumour. Constitutive JNK activity characterizes bovine T and B cells infected
21 with *T. parva*, and B cells and macrophages infected with *T. annulata*. Here, we show that *T.*
22 *annulata* manipulates JNK activation by recruiting JNK2, and not JNK1, to the parasite
23 surface, whereas JNK1 is found predominantly in the host cell nucleus. *In silico* analysis
24 identified 3 potential JNK-binding motifs in the previously characterized GPI-anchored
25 macroschizont surface protein (p104), and we demonstrate here that JNK2 is recruited to the
26 parasite via physical interaction with p104. A cell penetrating peptide harbouring a p104
27 JNK-binding motif also conserved in *T. parva* p104 competitively ablated binding,
28 whereupon liberated JNK2 became ubiquitinated and degraded. Sequestration of JNK2
29 depended on PKA-mediated phosphorylation of the conserved JNK-binding motif and upon
30 disruption of the p104/JNK2 complex loss of JNK2 resulted in diminished matrigel traversal
31 of *T. annulata*-transformed macrophages. Loss of JNK2 also resulted in upregulation of small
32 mitochondrial ARF that promoted autophagy consistent with cytosolic sequestration of JNK2
33 sustaining not only JNK2, but also nuclear JNK1 levels that combined contribute to both
34 survival and dissemination of *Theileria*-transformed macrophages.

35 **Author Summary**

36 *Theileria annulata* parasites infect and transform their host bovine leukocytes into tumour-
37 like cells that disseminate throughout infected animals causing a widespread disease called
38 tropical theileriosis. Virulence has been ascribed to the parasite's ability to constitutively
39 activate leukocyte c-Jun N-terminal Kinase (JNK) leading to permanent induction of Matrix
40 Metallo-Proteinase 9 (MMP9) that promotes transformed macrophage dissemination. In
41 attenuated live vaccines JNK-mediated AP-1-driven transcriptional activity is reduced so
42 dampening dissemination. However, in leukocytes JNK exists as two isoforms JNK1 and
43 JNK2 and here, we show for the first time that in *T. annulata*-transformed macrophages they
44 have different subcellular localisations and perform separate functions. Surprisingly, JNK2
45 associates with the parasite and is not in the nucleus like JNK1. JNK2 is hijacked by the
46 parasite and sequestered in a complex with a macroschizont surface protein called p104. Upon
47 forced complex dissociation JNK2 gets degraded and its loss negatively affects infected
48 macrophage survival and ability to disseminate.

49 **Introduction:**

50 In mammals, c-Jun-N-terminal kinase (JNK) is encoded by three genes, *mapk8*, *mapk9* and
51 *mapk10*: *mapk8* and *mapk9* code, respectively, for the ubiquitously expressed JNK1 and
52 JNK2 proteins, and *mapk10* codes for JNK3, whose expression is restricted to cardiac,
53 nervous and testicular tissues [1]. JNKs are activated by environmental stress including
54 extracellular insults such as radiation, redox, osmotic and temperature shocks, and
55 intracellular stress such as miss-folded proteins [1]. Biological responses transduced through
56 JNK-dependent pathways encompass proliferation, migration, survival, differentiation,
57 inflammation [1] and some of the JNK substrates participating in these processes have been
58 identified [2]. Changes in gene expression resulting from JNK activation may be accounted
59 for by the phosphorylation of several transcription factors and the ensuing alteration in their
60 transcriptional activity [2]. A well-characterized substrate of JNK is c-Jun, a component of the
61 AP-1 transcription factor that is essential for proliferation and cell survival. JNK can affect c-
62 Jun both positively and negatively: N-terminal phosphorylated c-Jun displays increased trans-
63 activating activity [3], whereas in neurons the E3 ubiquitin ligase SCFFbw7 specifically targets
64 phosphorylated c-Jun for proteasome degradation, thereby controlling the JNK/c-Jun
65 apoptotic pathway [4]. Additionally, in T lymphocytes c-Jun turnover is regulated by the E3
66 ubiquitin ligase Itch, whose activity increases upon JNK-dependent phosphorylation [5]. JNK
67 can promote cell motility via alteration of focal adhesion dynamics following JNK-mediated
68 phosphorylation of the focal adhesion adaptor paxillin [6-8].

69 Importantly, loss of *jnk2* in mouse embryonic fibroblasts (MEFs) increases cell proliferation,
70 whereas a loss of *jnk1* leads to decreased proliferation and these contrasting effects are
71 attributed to differential regulation of the mitogenic transcription factor c-Jun. JNK1 increases
72 c-Jun stability via phosphorylation of serine 73, whereas JNK2 decreases c-Jun stability by
73 promoting its ubiquitination [9, 10]. JNK2 also promotes ubiquitination-dependent

74 proteasomal degradation of small mitochondrial ARF (smARF), as in *jnk2*^{-/-} MEFs (Mouse
75 Embryonic Fibroblasts) levels of smARF rise to induce autophagy [11, 12]. SmARF is a short
76 isoform of the tumour suppressor p19^{ARF} and interestingly, suppression of smARF did not
77 require the kinase activity of JNK2 consistent with JNK2 acting as a scaffold protein [12].
78 The above examples highlight the disparate functions of JNK isoforms and underscore the
79 necessity to study them individually and together to properly grasp the cellular impact of JNK
80 activation.

81 Parasites of the genus *Theileria* are intracellular protozoans belonging to the *Apicomplexa*
82 phylum. *T. annulata* and *T. parva* are two particularly pathogenic species that cause bovine
83 lymphoproliferative diseases, respectively named tropical theileriosis and the East Coast
84 Fever (ECF). The target host cells of *T. parva* are T- and B-lymphocytes, whereas
85 monocytes/macrophages and B cells are preferentially infected by *T. annulata*. ECF and
86 tropical theileriosis display similarities to human lymphomas and myeloid leukemias. A live
87 attenuated vaccine exists to tropical theileriosis [13] that is generated by multiple passages of
88 infected monocytes/macrophages, which become attenuated having lost their hyper-
89 disseminating virulence trait [14]. *Theileria*-infected leukocytes behave as transformed cell
90 lines, as they no longer require exogenous growth or survival factors, can form colonies in
91 soft agar and give rise to disseminated tumours in immuno-compromised mice [15, 16].
92 Known as the only eukaryote pathogen to transform a eukaryote host cell *Theileria* achieves
93 this by manipulating host cell signalling pathways, reviewed in [17]. Several different
94 signalling pathways have been implicated, including TGF- β [18-20] and JNK kinase leading
95 to constitutive phosphorylation of c-Jun and activation of AP-1 [14, 21-23].

96 Remarkably, *Theileria*-induced transformation is strictly dependent on the presence of live
97 parasites, as the transformed host cell phenotype is fully reversible upon drug-induced
98 parasite death; drug-treated transformed leukocytes return to a quiescent, non-activated state,

99 and eventually die [24]. *Theileria*-dependent JNK1 activity is required for survival of *T. parva*
100 transformed B lymphocytes, as demonstrated by over expression of a dominant negative
101 kinase-dead mutant of JNK1 and/or via the use of pan JNK inhibitor [16, 25]. While the
102 parasite-derived molecular mechanism(s) underlying JNK activation is/are unknown, JNK1-
103 mediated survival of *Theileria*-transformed leukocytes has been attributed to AP-1-driven
104 expression of the anti-apoptotic genes *Mcl-1* and *c-IAP* [16], and uncontrolled proliferation
105 linked to AP-1-driven expression of transferrin receptor and cyclin D1 [23].

106 One of the characteristics of *Theileria*-transformed leukocytes is they display heightened
107 oxidative stress due in part to uncontrolled proliferation-related production of H₂O₂ [26, 27].
108 This raises the possibility that exposure to H₂O₂ contributes to induction of JNK activity, as
109 JNK activation occurs in response to stress. Taken together, JNK activation seems a key event
110 in *Theileria*-induced leukocyte transformation and the aim of this study was to examine
111 whether *Theileria* infection affects differentially JNK1 versus JNK2 and do the two isoforms
112 play similar or different roles in parasite-induced leukocyte transformation.

113 **Results:**

114 **Cytosolic localization of JNK2 versus nuclear localization of JNK1 in *Theileria*-infected
115 macrophages:**

116 To understand how the two major JNK isoforms participate in *Theileria*-induced leukocyte
117 transformation we ascertained the sub-cellular distribution of JNK1 versus JNK2 in *Theileria*-
118 infected macrophages. JNK1 partitions into the cytosolic and nuclear fractions and expression
119 levels are parasite-dependent, decreasing upon Bw720c-induced parasite death, and this is
120 particularly obvious for nuclear JNK1 (Figure 1A, left). In contrast to JNK1, JNK2 partitions
121 principally in the cytosolic fraction and again levels decrease upon Bw720c (Fig 1B).

122 Immunofluorescence analysis revealed JNK2 in the cytosol, decorating the intracellular
123 macroschizont highlighted with a monoclonal antibody (1C12) to *T. annulata* p104 [28].

124 ***T. annulata* p104 is a putative macroschizont surface JNK-binding protein.**

125 As JNK2 appears associated with the macroschizont we searched for a parasite surface protein
126 predicted to have a JNK-binding motif [2]. *In silico* analyses were performed on three
127 different species of *Theileria* (*T. annulata*, *T. parva* and *T. orientalis*). A Dfinder scan of the
128 whole predicted proteomes of three *Theileria* species in search of D-motifs [29] led to
129 identification of 26, 24 and 25 proteins, respectively (S1 file). Next, we asked which of these
130 75 putative *Theileria* JNK-binding proteins also had a predicted signal peptide and this
131 criterion identified only one protein in *T. annulata* (TA08425), two proteins in *T. parva*
132 (TP04_0437 orthologue of TA08425, and a hypothetical protein TP02_0553) and no protein
133 in *T. orientalis*. TA08425 codes for a GPI-anchored *T. annulata* macroschizont surface
134 protein called p104 [28] that has 3 putative decapeptide D-motifs KNESMLRLDL,
135 KSPKRPESLD and KRSKSFDDLT located between amino acids 331-341, 606-616 and 804-
136 814, respectively. However, only the decapeptide motif between amino acids 804 and 814 is
137 conserved in the *T. parva* p104 orthologue (TP04_0437). The amino acid sequence in this
138 region of p104 is not conserved in the non-transforming *T. orientalis* (TOT_040000478).

139 We examined therefore, whether the conserved D-motif (KRSKSFDDLT) mediated JNK2
140 binding to the *T. annulata* macroschizont surface protein p104. First, a pan-JNK antibody
141 precipitated endogenous p104 from extracts of *T. annulata*-infected TBL3 B cells, but not
142 from uninfected BL3 B cells (Figure 2A). As expected (see Fig 1), p104 was preferentially
143 found in pan JNK precipitates (Fig 2B, left), and specifically in JNK2 precipitates (Fig 2B,
144 right and Fig 3B). Altogether these results suggest that JNK2 is associated with p104 at the
145 surface of the *Theileria* macroschizont.

146 **Phosphorylation of the JNK-binding motif increases the affinity of p104 for JNK2.**

147 To understand the consequences of JNK2 association with p104, we specifically ablated their
148 interaction. Located on the macroschizont surface GPI-anchored p104 has been described as
149 being phosphorylated *in vivo* at several sites [30]. We noticed that two phosphorylated
150 residues occurred in the conserved JNK-binding motif, specifically phospho-S806 and –S808
151 (TA08425 numbering). Consequently, penetrating peptides harbouring the conserved p104
152 decapeptide JNK-binding motif and a mutant peptide, where S806 and S808 had been
153 replaced by alanine, together with an irrelevant peptide were synthesized (Table 1).

154 **Table 1. Synthesized peptides harbouring JNK binding motif.**

Peptides	Sequences	Phospho-epitope	Predicted kinases phosphorylation sites
P (peptide with conserved JNK-binding motif)	HVKKKKIKREIKITGKIVKL <u>KRS<u>KSFDDLTTK</u></u> (FITC)	S806, S808	PKA
mP (mutant peptide)	HVKKKKIKREIKITGKIVKL <u>KRA<u>KAFDDLTTK</u></u> (FITC)	Mutations of S806, S808 in alanine	PKA
IrrP (irrelevant peptide)	HVKKKKIKREIKIAAGRYGRELRRMADEFHV-K (FITC)		

155 **Legend Table 1.** The different FITC-labelled penetrating peptides synthesized. (P) is the
156 conserved wild type amino acid sequence with S806 and S808 shown underlined. The mutant
157 peptide (mP) has S806 and S808 changed to A806 and A808 (underlined). An irrelevant
158 peptide (IrrP) used as a negative control to compete for JNK-binding to p104. The JNK-
159 binding motif is in bold and the sequence of the fused penetrating peptide indicated at the N-
160 terminus.

161 All peptides were FITC conjugated and penetration into *T. annulata*-infected macrophages
162 confirmed by immunofluorescence (FigS1). The peptide (P) corresponding to the wild type
163 JNK-binding motif was able to ablate in dose-dependent manner JNK2 association with p104
164 (Figure 3). Importantly, the mutant (S>A) peptide (mP) did not abrogate JNK2 binding to
165 p104 (Fig 3A, tracks 6 & 7), consistent with phosphorylation of S806 and/or S808 promoting
166 p104 binding to JNK2. Following peptide-mediated abrogation of the JNK2/p104 complex
167 the level of p54 JNK2 decreased, due to ubiquination of JNK2, but no drop in JNK2 levels
168 was observed following proteasome blockade by MG132 (Fig 3A tracks 4 & 5 and Fig 4A).
169 Although peptide-induced complex disruption reduced JNK2 levels no effect was observed on
170 cytosolic JNK1 (Fig 4B), but loss of cytosolic JNK2 led to an increase in the amount of
171 nuclear JNK1 (Fig 4B).

172 **PKA, but not JNK kinase activity contributes to p104 association with JNK2.**

173 Both S806 and S808 in p104 occur in a context (KRS*KS*FD) consistent with them being
174 potentially phosphorylated by PKA [28]. Consequently, *T. annulata*-infected macrophages
175 were treated for 2 h with myristoylated PKI or the ATP analogue H89 and both treatments
176 dampened the association of p104 with JNK2 (Figure 5A). By contrast, treatment with a pan
177 JNK inhibitor (pan-JNKi), or a JNK2-specific inhibitor (JNK2i) did not alter the ability of
178 p104 to bind JNK2 (Fig 5B). We conclude that cAMP-dependent PKA likely phosphorylates
179 S806 and/or S808 and their phosphorylation favours binding of p104 to bind JNK2. The
180 kinase activity of JNK2 does not appear necessary for complex formation, leaving open that
181 JNK2 could have a scaffold function (see below).

182 **Abrogation of JNK2/p104 association reduces matrigel traversal of *Theileria*-
183 transformed macrophages:**

184 Matrigel traversal is used as a measure of dissemination potential and virulent (V) *T.* *185 annulata*-transformed macrophages traverse matrigel better than attenuated (A) macrophages
186 [20]. Matrigel transversal is significantly decreased when *T. annulata*-transformed
187 macrophages are treated with the wild type JNK-binding motif peptide (P), whereas the (S>A)
188 mutant peptide mP and the irrelevant peptide (irrP) had no significant effect (Figure 6A). It is
189 well established that *Theileria*-infected macrophages are characterised by AP-1-driven
190 transcription of *mmp9* and increased MMP9 activity promotes matrigel traversal and
191 dissemination [21, 31, 32]. JNK2 association with p104 was therefore ablated and loss of
192 MMP9 activity revealed by gelatin gel assay (Fig 6B, left). Peptide-induced disruption of
193 JNK2/p104 binding slightly, but significantly, inhibited *mmp9* transcription as estimated by
194 qRT-PCR (Fig 6B, right). Peptide-induced complex dissociation also specifically reduced
195 nuclear c-Jun phosphorylation (Fig 6C) consistent with the drop in nuclear JNK1 levels (Fig
196 4B).

197 **Abrogation of the JNK2/p104 association leads to upregulation of ARF levels**

198 Because a non-kinase, scaffold protein function for JNK2 has been described to regulate
199 smARF levels and induce autophagy [33], we monitored ARF levels following disruption of
200 the p104/JNK2 complex and subsequent JNK2 degradation (Figure 7). Loss of JNK2
201 provoked by peptide-treatment resulted in upregulation of p14ARF. The rise in ARF levels
202 was accompanied an increase in amounts of processed autophagosome membrane protein
203 LC3B-II and appearance of LC3B-II-positive foci (Figs 7A low panel, and B).

204 **Discussion:**

205 Constitutively active JNK1 has a nuclear localisation that is dependent on live *T. annulata*
206 macroschizonts, since nuclear JNK1 levels were ablated upon drug (Bw720c)-induced
207 parasite death. By contrast, JNK2 is mainly in the infected macrophage cytosol associated
208 with the macroschizont with only a minor fraction of JNK2 being nuclear and cytosolic JNK2
209 levels also depend on the live parasite being present (Figure 1). Simply by their different
210 subcellular localisations one can surmise that JNK1 and JNK2 likely play different roles in
211 *Theileria*-induced transformation of host leukocytes.

212 The close association between JNK2 and the parasite revealed by immunofluorescence led us
213 to search for a parasite encoded JNK-binding protein located on the macroschizont surface.
214 Interrogating the predicted *Theileria* proteomes with a consensus D-motif revealed the
215 presence of 3 potential JNK-binding motifs in the GPI-anchored macroschizont *T. annulata*
216 protein known as p104 [28]. We decided to characterise the putative JNK-binding site located
217 between amino acids 804 to 814, as this is conserved in the *T. parva* p104 orthologue
218 (TP04_0437), and absent in the non-transforming *T. orientalis* (TOT_040000478) protein.
219 Although not characterised here it remains possible that one or both of the other 2 *T. annulata*
220 p104-specific D-motifs might also contribute to species-specific JNK2 binding by p104. *In*
221 *vitro* recombinant *T. annulata* p104 clearly binds to recombinant JNK2 (FigS2A) making it
222 possible to study the contribution of the 2 other D-motifs to species-specific JNK2 binding.

223 The conserved JNK-binding motif in p104 is distal to the previously described EB1-binding
224 motif (SKIP) that's located between amino acids 566 and 569 [28]. It is highly unlikely
225 therefore that the JNK-binding motif penetrating peptide competes for EB1-binding.
226 Furthermore, EB1 co-localizes with p104 on the macroschizont surface in a cell-cycle
227 dependent manner, being more pronounced during cell division [28]. This contrasts with

228 JNK2-binding to p104 that does not require division of infected macrophages. Although p104
229 acts as an EB1-binding protein attempts to interfere with EB1 binding to the macroschizont
230 surface failed [28]. One explanation could be that JNK2 binding to p104 creates a platform
231 favourable to EB1 association with the macroschizont during host cell mitosis. As we have
232 shown that PKA can phosphorylate *in vitro* p104 (Fig S2B) this strongly argues that *in vivo*
233 [30] PKA-mediated phosphorylation of p104 promotes association with JNK2 and indeed
234 PKA inhibition reduced the amount of p104 detected in JNK2 immunoprecipitates (Fig 5).
235 This implies that the complex at the surface of the macroschizont contains not only p104 and
236 JNK2, but also PKA. By contrast, Cdk1-mediated phosphorylation of p104 seems to play a
237 role in EB1 binding during mitosis, although the role of other kinases in regulating the
238 interaction between p104 and EB1 has not been ruled out [28].

239 Importantly, both S806 and S808 have previously described as being phosphorylated *in vivo*
240 [30], so the strategy we adopted to elucidate the role of the JNK2/p104 complex was to use a
241 penetrating peptide harbouring the conserved JNK-binding motif, as a competitive p104
242 substrate for PKA. When S806 and S808 are changed to A806 and A808 the mutant
243 penetrating peptide is no longer a competitive substrate for PKA and is incapable of
244 disrupting JNK2 binding to p104. It's noteworthy that binding of JNK2 to p104 *in vivo* was
245 disrupted by inhibition of PKA activity by two independent PKA inhibitors, myristolated
246 PKI and H89. Interestingly however, recruitment of JNK2 to p104 was insensitive to
247 inhibition of JNK kinase activity suggesting JNK2 acts as a scaffold protein on which the
248 complex is assembled at the macroschizont surface.

249 We focused on JNK2 to gain insights as to what might be the physiological advantage to
250 *Theileria*-induced leukocyte transformation in retaining JNK2 in the infected host cell cytosol
251 sequestered at the macroschizont surface. The ensembles of our results pinpoint at least 3
252 advantageous consequences of p104-mediated JNK2 sequestration: 1) While associated with

253 the macroschizont surface JNK2 is protected from ubiquitination and proteasomal degradation
254 and sustained JNK2 levels suppress smARF-mediated autophagy [11, 12]. As such JNK2
255 sequestration contributes to *Theileria*-infected leukocyte survival and indeed, 24 h following
256 peptide-induced JNK2 degradation infected macrophages become Annexin V-positive (Fig
257 S3). 2) Since nuclear JNK2 decreases c-Jun stability by promoting its ubiquitination [9, 10],
258 cytosolic retention of JNK2 could contribute to sustained nuclear c-Jun levels perhaps by
259 preventing DET1 mediated ubiquitination [34]. 3) Now, we show that peptide-induced
260 complex dissociation led to JNK2 ubiquitination and degradation and a loss of nuclear c-Jun
261 fluorescence (Fig 6C). Moreover, upon loss of cytosolic JNK2 the nuclear levels of JNK1 also
262 decrease suggesting an alternative reason for loss of c-Jun phosphorylation (Fig 4B).
263 Dampening of nuclear JNK1 levels also likely explains the drop in *mmp9* transcription and
264 reduced matrigel traversal (Fig 6).

265 The macroschizont surface of *T. annulata*-infected macrophages is known to recruit another
266 host cell tumour suppressor p53, preventing its translocation to the nucleus, inhibiting p53-
267 mediated apoptosis, and thus contributing to host cell survival [35]. Moreover, ARF
268 participates in the regulation of p53 interaction with MDM2 [36-38]. It's interesting to note
269 that antisense knockdown of *jnk2* has been described to dampen phosphorylation of p53 [39]
270 making it possible that p53 is a substrate of JNK2 at the macroschizont surface in *Theileria*-
271 infected leukocytes. It remains to be seen whether macroschizont recruitment of the IKK-
272 signalosome [40] also involves binding to JNK2/p104, or since the number of parasite-
273 associated IKK signalosomes fluctuates in the course of the host cell cycle, binding occurs
274 indirectly perhaps via EB1, or other cytoskeleton-associated proteins.

275 Infection by another apicomplexan parasite *Toxoplasma gondii* leads to constitutive activation
276 of a MAP/SAP kinase called p38 [41]. This contrasts with *Theileria*, where in different types
277 of leukocytes transformed either by *T. annulata* or *T. parva* infection leads to constitutive

278 activation of JNK rather than p38 [22, 42, 43]. Just why JNK2, and not JNK1 binds to p104 *in*
279 *vivo* is not clear, since the JNK-binding motifs identified in p104 do not in principal
280 discriminate between JNK isoforms. One possibility is that *in vivo* PKA-mediated
281 phosphorylation of the conserved D-motif renders it more specific for JNK2 over JNK1. The
282 *T. gondii* p38-binding protein harbours 2 MAP-kinase binding motifs, called KIM1 and KIM2
283 for Kinase-Interaction-Motifs (also known as D-motifs) that occur in a disordered C-terminal
284 repetitive region of GR24 [44]. Although the MAP-kinase binding sites in p104 and GRA24
285 fit the same loose D-motif consensus their amino acid sequences are different. The 2 D-motifs
286 present in GRA24 combine to provoke high affinity binding of p38, whereas we posit that
287 PKA-mediated phosphorylation of S806 & S808 in p104 promotes binding to JNK2. It's
288 remarkable that these two pathogenic *Apicomplexa* both manipulate host cell MAP/SAP
289 kinase signalling, but do so in different ways. Secreted GR24 goes into the host cell nucleus,
290 binds and activates p38, whereas GPI-anchored p104 expressed on the macroschizont surface
291 binds JNK2 preventing it from translocating to the nucleus, while activated JNK1 goes to the
292 nucleus and phosphorylates c-Jun to drive *mmp9* transcription. Clearly, the need for a better
293 understanding of both kinase and non-kinase, scaffold-like functions of JNK2 bound to p104
294 will animate future studies aimed at dissecting *Theileria*-induced leukocyte transformation.

295 **Materials and methods:**

296 **Chemicals and reagents.** Pan-JNK inhibitor (JNK II #420128, Calbiochem, LA JOLLA) was
297 added at 16 μ M and JNK2 inhibitor (JNK IX: # 420136, Calbiochem LA JOLLA) was added
298 at 50 nM. Synthetized penetrating peptides harbouring JNK binding domain were produced
299 by GL Biochem Ltd (Shanghai, China) and was added at 1 μ M or 5 μ M for 2 h. MG132
300 (CAS 1211877-36-9) was added at 10 μ M for 2 h. PKA inhibitor H89 (SIGMA-Aldrich) was
301 added at 10 μ M for 2 h and Myr PKI inhibitor (SIGMA-Aldrich) added at 50 μ M for 2 h.

302 ***Theileria annulata*-infected macrophage culture.** *T. annulata*-infected
303 monocytes/macrophages used in this study are the Ode virulent corresponding to passage 53
304 [45]. All cells were incubated at 37°C with 5% CO₂ in Roswell Park Memorial Institute
305 medium (RPMI) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine,
306 100 U penicillin, 0.1 mg/ml streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic
307 acid (HEPES).

308 **Analyses of JNK binding sites in *Theileria* proteomes.** We obtained the protein sequences
309 of *Theileria annulata*, *T. parva* and *T. orientalis* from PiroplasmDB. We used ‘dfinder’
310 programme, with default settings [46] to scan the complete predicted proteomes of the three
311 species in search of D-sites (docking site for JNK). The search was filtered with cutoff
312 threshold of 1e⁻²³ as recommended [46]. Signal Peptide prediction was done by TOPCONS
313 [47].

314 **Antibodies and western blot analyses.** Cells were harvested and lysed by using lysis buffer
315 (20 mM HEPES, 1% Nonidet P-40 [NP-40], 0.1% SDS, 150 mM NaCl, 2 mM EDTA,
316 phosphatase inhibitor cocktail tablet (PhosSTOP; Roche), and protease inhibitor cocktail
317 tablet (Complete Mini EDTA free; Roche). The protein concentration was determined by the
318 Bradford protein assay. Cell lysates were subjected to Western blot analysis using
319 conventional SDS-PAGE and protein transfer onto nitrocellulose filters (Protran and
320 Whatman). Western blotting was performed as described previously [20]. The membrane was
321 blocked with a solution containing 5% of BSA and Tris-buffered saline–Tween (TBST) for 1
322 h. The anti-*T. annulata* antibodies used and diluted in the blocking solution were the 1C12
323 monoclonal antibody against p104 [48] and an antibody to ribonucleotide reductase [49].
324 Polyclonal anti-JNK (sc-571), polyclonal anti-JNK2 (sc-46013), monoclonal anti-JNK2 (sc-
325 271133), monoclonal anti-ubiquitine (sc-271289), polyclonal anti-phospho-c-Jun (sc-7981)
326 and a polyclonal anti-p14^{ARF} (sc-8340) were purchased from Santa Cruz Biotechnologies

327 (Santa Cruz, CA, USA), anti-LC3B-II (NB600-1384) from Novus Biologicals, the anti-PARP
328 antibody (ab194586) from Abcam (Abcam PLC, Cambridge) and anti-MMP9 (AV33090)
329 from Sigma. After washing, proteins were visualized with ECL western blotting detection
330 reagents (Thermo Scientific) on fusion FX (Vilber Lourmat). The β-actin level was used as a
331 loading control throughout.

332 **Immunofluorescence analysis:** Ode macrophages were treated or not with peptide and fixed
333 in buffer containing 4% paraformaldehyde and 3% of sucrose for 15 min. Permeabilization
334 was performed using 0.01% Triton in phosphate-buffered saline medium for 5 min followed
335 by two washes with 1X phosphate-buffered saline. Cells were then blocked with 3% bovine
336 serum albumin for 1 h, stained with the anti-LC3B-II antibody, or the anti-phospho-c-Jun for
337 120 min at room temperature, and washed 3 times with buffer before incubation with a
338 secondary anti-rabbit IgG antibody conjugated with respectively Alexa 546 or Alexa 647
339 (Molecular Probes) in darkness for 60 min at room temperature. Cells were stained with 4',6-
340 diamidino-2-phenylindole (Bibenzimide H 33258, Sigma) for nucleus labeling. Dako
341 mounting medium was used (Glostrup, Denmark). The immunolabeled cells were examined
342 with a Zeiss observer Z1, camera QICAM.

343 **Co-immunoprecipitation.** *T. annulata*-infected macrophages were harvested and lysed in the
344 lysis buffer containing 20 mM HEPES, 1% Nonidet P-40 [NP-40], 0.1% SDS, 150 mM NaCl,
345 2 mM EDTA, phosphatase inhibitor cocktail tablet (PhosSTOP; Roche), and protease
346 inhibitor cocktail tablet (Complete Mini EDTA free; Roche). The protein concentration was
347 determined by the Bradford protein assay. Protein-G Dynabeads (Invitrogen) were washed
348 twice with PBS1X solution. After incubation with the antibody of interest for 2.5 h, 500 µg of
349 protein extracts were added overnight. The beads were washed 5-times with lysis buffer
350 supplemented with proteases and phosphatases inhibitors and boiled in Laemmli buffer before
351 performing western blotting.

352 **Matrigel chambers assay.** The invasive capacity of *Theileria*-infected macrophages was
353 assessed *in vitro* using matrigel migration chambers [16]. Culture coat 96-well medium BME
354 cell invasion assay was obtained from Culturex instructions (3482-096-K). After 24 h of
355 incubation at 37°C, each well of the top chamber was washed once in buffer. The top chamber
356 was placed back on the receiver plate. 100µl of cell dissociation solution/Calcein AM were
357 added to the bottom chamber of each well, incubated at 37°C for 1 h to fluorescently label
358 cells and dissociate them from the membrane before reading at 485 nm excitation, 520 nm
359 emission using the same parameters as the standard curve.

360 **Zymography (gelatin gel assay)**

361 We used 10% SDS-Polyacrylamide gel electrophoresis containing 1% co-polymerized
362 gelatin to detect secreted gelatinases such as MMP2 and MMP9. 5x10⁶ cells were washed
363 three times with cold PBS to remove all the serum and were plated in 6-well plates in 5ml
364 serum free culture medium. Supernatants from these cultures were collected after 24 h.
365 Supernatant samples were mixed with 2X sample buffer containing 0.5M Tris-HCl pH 6.8,
366 20% Glycerol, 10% SDS and 0.005% Bromophenol Blue. They were left at room temperature
367 for 10 min and then loaded onto the gel. Migration was performed in 1X Tris-Glycine SDS
368 Running buffer at 125V. The gels were washed twice for 30 min in renaturing buffer
369 containing 2.5% Triton X-100, which removed the SDS. To activate the proteases, gels were
370 incubated at 37°C for 18 h in 30ml of a solution containing 50mM Tris-HCl pH 7.6, 5mM
371 CaCl₂ and 0.02% Triton X-100. Gels were subsequently stained for 2 h with a solution
372 containing 0.5% Coomasie Blue R-250, 40% Methanol, 10% Acetic acid and de-stained with
373 50% Methanol, 10% Acetic Acid. Areas of digestion appeared as clear bands, against a darkly
374 stained background due to the substrate being degraded by the enzyme.

375 **RNA extraction, reverse transcription and qRT-PCR:** Total RNA was extracted from cells
376 with the RNeasy® Plus mini kit (QIAGEN) and quantified by the NanoDrop ND1000

377 Spectrophotometer. cDNA was synthesized from 1000 ng of RNA by using M-MLV reverse
378 transcriptase enzyme (Promega). The qRT-PCR reaction mixture included 2.5 μ l Absolute
379 blue qPCR SYBR green (Thermo Scientific), 0.5 μ l of each forward and reverse primers, 4 μ l
380 molecular grade water, and 2.5 μ l of 1:20 diluted cDNA.

381 Actin left primer: AGAGGCATCCTGACCCTCAA;

382 Actin right primer: TCTCCATGTCGTCCCAGTTG;

383 MMP9 left: TGGCACGGAGGTGTGATCTA;

384 MMP9 right: GACAAGAAGTGGGGCTTCTG.

385 **GST- pull downs:** The C-terminal disordered region (504-839) of *T. annulata* p104 was
386 subcloned into a modified pET vector enabling expression of GST fusion proteins with a C-
387 terminal hexa-histidine tag by PCR. The cDNA of full-length human JNK2 was subcloned
388 into another modified pET plasmid allowing the expression of proteins with an N-terminal
389 hexahistidine tag. All protein constructs were expressed in *Escherichia coli* Rosetta (DE)
390 pLysS cells with standard techniques. Protein expression was induced at 25 °C for 3 h by
391 adding 0.2 mM IPTG, cells were lysed, and the lysate was loaded onto Ni-NTA resin and
392 eluted by imidazol. GST-p104 samples were then loaded to glutathione resin and washed with
393 GST wash buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.05 % IGEPAL, 1mM EDTA and 5
394 mM beta-mercaptoethanol). Ni-NTA eluted JNK2 was further purified by using an ion-
395 exchange column (resourceQ) and was eluted with a salt gradient (0.1M-1M NaCl). In a
396 typical GST-pull down experiment 50 μ l of glutathione resin loaded with the bait was
397 incubated in 100 μ M of JNK2 solution in GST wash buffer and washed three times. After
398 addition of SDS loading buffer the resin was subjected to SDS-PAGE and gels were stained
399 by Coomassie Brilliant Blue protein dye, or the gels were subjected to Western-blots using
400 anti-His, GE Healthcare (27-4710-01), or anti-JNK, Cell Signaling (3708S), antibodies

401 according to the supplier's recommendations. All plasmid DNA sequences were confirmed by
402 sequencing. GST protein with a C-terminal hexa-histidine tag was used as the negative
403 control for the GST-pull down experiments.

404 ***In vitro* kinase assays:** The catalytic domain of PKA with an N-terminal hexa-histidine tag
405 was expressed in *E. coli* using the pET15b PKA Cat vector [50] and purified with Ni-NTA
406 resin similarly as described above. 0.5 μ M PKA catalytic subunit was incubated with 5 μ M
407 GST or GST-p104 C-terminal disordered region (504-83 9) fusion protein in the presence of
408 radioactively labeled ATP(γ)P³² (~5 μ Ci). Aliquots of the kinase reactions were taken at
409 different time points and run on SDS-PAGE. Gels were mounted onto filter paper, dried and
410 subjected to phosphoimaging using a Typhoon Trio+ scanner (GE Healthcare). The kinase
411 buffer contained 20 mM Tris pH, 100 mM NaCl, 0.05 % IGEPAL, 5 % glycerol, 2 mM
412 TCEP, 5 mM MgCl₂ and 0.25 mM ATP.

413 **Flow cytometry:** Infected macrophages were treated with 5 μ M of non-constrained CCP. 10⁶
414 of Ode cells are prepared in 1 ml of PBS with 10% FBS in each test tube. After a
415 centrifugation for 5 min at 200 \times g and 4 °C, cells are resuspended in 100 μ l annexin V
416 Binding buffer. 5 μ l of annexin V and 5 μ l of 7AAD (7-aminoactinomycin D) are added to
417 each tube except single stained control. Ode are incubated 15 min in the dark at room
418 temperature with 400 μ l ice cold annexin V binding buffer and then analyzed on the flow
419 cytometry (Accuri C6 – C flow Plus software).

420 **Statistical analysis.** Experiments were performed at least three times and results presented as
421 mean values +/- SEM. p values were determined using the Student's t-test. Results were
422 considered significant for p <0.05.

423 **Acknowledgments:**

424 We would like to thank Professor Brian Shiels for gift of antibodies to *Theileria* p104 (mAb
425 1C12).

426 **References**

- 427 1. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell*.
428 2000;103(2):239-52. PubMed PMID: 11057897.
- 429 2. Bogoyevitch MA, Kobe B. Uses for JNK: the many and varied substrates of the c-Jun
430 N-terminal kinases. *Microbiol Mol Biol Rev*. 2006;70(4):1061-95. doi:
431 10.1128/MMBR.00025-06. PubMed PMID: 17158707; PubMed Central PMCID:
432 PMCPMC1698509.
- 433 3. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and
434 UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes*
435 *Dev*. 1993;7(11):2135-48. PubMed PMID: 8224842.
- 436 4. Nateri AS, Riera-Sans L, Da Costa C, Behrens A. The ubiquitin ligase SCFFbw7
437 antagonizes apoptotic JNK signaling. *Science*. 2004;303(5662):1374-8. doi:
438 10.1126/science.1092880. PubMed PMID: 14739463.
- 439 5. Gao M, Labuda T, Xia Y, Gallagher E, Fang D, Liu YC, et al. Jun turnover is
440 controlled through JNK-dependent phosphorylation of the E3 ligase Itch. *Science*.
441 2004;306(5694):271-5. doi: 10.1126/science.1099414. PubMed PMID: 15358865.
- 442 6. Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates
443 paxillin and regulates cell migration. *Nature*. 2003;424(6945):219-23. doi:
444 10.1038/nature01745. PubMed PMID: 12853963.
- 445 7. Ueno H, Tomiyama A, Yamaguchi H, Uekita T, Shirakihara T, Nakashima K, et al.
446 Augmentation of invadopodia formation in temozolomide-resistant or adopted glioma is
447 regulated by c-Jun terminal kinase-paxillin axis. *Biochem Biophys Res Commun*.
448 2015;468(1-2):240-7. doi: 10.1016/j.bbrc.2015.10.122. PubMed PMID: 26518652.
- 449 8. Wang C, Zhao Y, Su Y, Li R, Lin Y, Zhou X, et al. C-Jun N-terminal kinase (JNK)
450 mediates Wnt5a-induced cell motility dependent or independent of RhoA pathway in human
451 dental papilla cells. *PLoS One*. 2013;8(7):e69440. doi: 10.1371/journal.pone.0069440.
452 PubMed PMID: 23844260; PubMed Central PMCID: PMCPMC3700942.
- 453 9. Bode AM, Dong Z. The functional contrariety of JNK. *Mol Carcinog*. 2007;46(8):591-
454 8. doi: 10.1002/mc.20348. PubMed PMID: 17538955; PubMed Central PMCID:
455 PMCPMC2832829.
- 456 10. Fuchs SY, Dolan L, Davis RJ, Ronai Z. Phosphorylation-dependent targeting of c-Jun
457 ubiquitination by Jun N-kinase. *Oncogene*. 1996;13(7):1531-5. PubMed PMID: 8875991.
- 458 11. Budina-Kolomets A, Hontz RD, Pimkina J, Murphy ME. A conserved domain in exon
459 2 coding for the human and murine ARF tumor suppressor protein is required for autophagy
460 induction. *Autophagy*. 2013;9(10):1553-65. doi: 10.4161/auto.25831. PubMed PMID:
461 23939042; PubMed Central PMCID: PMCPMC4623555.
- 462 12. Zhang Q, Kuang H, Chen C, Yan J, Do-Umehara HC, Liu XY, et al. The kinase Jnk2
463 promotes stress-induced mitophagy by targeting the small mitochondrial form of the tumor
464 suppressor ARF for degradation. *Nat Immunol*. 2015;16(5):458-66. doi: 10.1038/ni.3130.
465 PubMed PMID: 25799126; PubMed Central PMCID: PMCPMC4451949.
- 466 13. Darghouth MA. Review on the experience with live attenuated vaccines against
467 tropical theileriosis in Tunisia: considerations for the present and implications for the future.
468 *Vaccine*. 2008;26 Suppl 6:G4-G10. doi: 10.1016/j.vaccine.2008.09.065. PubMed PMID:
469 19178892.

470 14. Baylis HA, Megson A, Hall R. Infection with *Theileria annulata* induces expression of
471 matrix metalloproteinase 9 and transcription factor AP-1 in bovine leucocytes. *Mol Biochem*
472 *Parasitol*. 1995;69(2):211-22. PubMed PMID: 7770085.

473 15. Fell AH, Preston PM, Ansell JD. Establishment of *Theileria*-infected bovine cell lines
474 in scid mice. *Parasite Immunol*. 1990;12(3):335-9. PubMed PMID: 2117267.

475 16. Lizundia R, Chaussepied M, Huerre M, Werling D, Di Santo JP, Langsley G. c-Jun
476 NH2-terminal kinase/c-Jun signaling promotes survival and metastasis of B lymphocytes
477 transformed by *Theileria*. *Cancer Res*. 2006;66(12):6105-10. doi: 10.1158/0008-5472.CAN-
478 05-3861. PubMed PMID: 16778183.

479 17. Shiels B, Langsley G, Weir W, Pain A, McKellar S, Dobbelaere D. Alteration of host
480 cell phenotype by *Theileria annulata* and *Theileria parva*: mining for manipulators in the
481 parasite genomes. *Int J Parasitol*. 2006;36(1):9-21. doi: 10.1016/j.ijpara.2005.09.002. PubMed
482 PMID: 16221473.

483 18. Chaussepied M, Janski N, Baumgartner M, Lizundia R, Jensen K, Weir W, et al. TGF-
484 b2 induction regulates invasiveness of *Theileria*-transformed leukocytes and disease
485 susceptibility. *PLoS Pathog*. 2010;6(11):e1001197. doi: 10.1371/journal.ppat.1001197.
486 PubMed PMID: 21124992; PubMed Central PMCID: PMCPMC2987823.

487 19. Haidar M, Echebli N, Ding Y, Kamau E, Langsley G. Transforming growth factor
488 beta2 promotes transcription of COX2 and EP4, leading to a prostaglandin E2-driven
489 autostimulatory loop that enhances virulence of *Theileria annulata*-transformed macrophages.
490 *Infect Immun*. 2015;83(5):1869-80. doi: 10.1128/IAI.02975-14. PubMed PMID: 25690101;
491 PubMed Central PMCID: PMCPMC4399038.

492 20. Haidar M, Whitworth J, Noe G, Liu WQ, Vidal M, Langsley G. TGF-beta2 induces
493 Grb2 to recruit PI3-K to TGF-RII that activates JNK/AP-1-signaling and augments
494 invasiveness of *Theileria*-transformed macrophages. *Sci Rep*. 2015;5:15688. doi:
495 10.1038/srep15688. PubMed PMID: 26511382; PubMed Central PMCID:
496 PMCPMC4625156.

497 21. Adamson R, Logan M, Kinnaird J, Langsley G, Hall R. Loss of matrix
498 metalloproteinase 9 activity in *Theileria annulata*-attenuated cells is at the transcriptional level
499 and is associated with differentially expressed AP-1 species. *Mol Biochem Parasitol*.
500 2000;106(1):51-61. PubMed PMID: 10743610.

501 22. Chaussepied M, Lallemand D, Moreau MF, Adamson R, Hall R, Langsley G. Upregulation
502 of Jun and Fos family members and permanent JNK activity lead to constitutive
503 AP-1 activation in *Theileria*-transformed leukocytes. *Mol Biochem Parasitol*. 1998;94(2):215-
504 26. PubMed PMID: 9747972.

505 23. Lizundia R, Chaussepied M, Naissant B, Masse GX, Quevillon E, Michel F, et al. The
506 JNK/AP-1 pathway upregulates expression of the recycling endosome rab11a gene in B cells
507 transformed by *Theileria*. *Cell Microbiol*. 2007;9(8):1936-45. doi: 10.1111/j.1462-
508 5822.2007.00925.x. PubMed PMID: 17388783.

509 24. Dobbelaere D, Heussler V. Transformation of leukocytes by *Theileria parva* and *T.*
510 *annulata*. *Annu Rev Microbiol*. 1999;53:1-42. doi: 10.1146/annurev.micro.53.1.1. PubMed
511 PMID: 10547684.

512 25. Lizundia R, Sengmanivong L, Guergnon J, Muller T, Schnelle T, Langsley G, et al. Use
513 of micro-rotation imaging to study JNK-mediated cell survival in *Theileria parva*-infected
514 B-lymphocytes. *Parasitology*. 2005;130(Pt 6):629-35. PubMed PMID: 15977899.

515 26. Metheni M, Echebli N, Chaussepied M, Ransy C, Chereau C, Jensen K, et al. The
516 level of H(2)O(2) type oxidative stress regulates virulence of *Theileria*-transformed
517 leukocytes. *Cell Microbiol*. 2014;16(2):269-79. doi: 10.1111/cmi.12218. PubMed PMID:
518 24112286; PubMed Central PMCID: PMCPMC3906831.

519 27. Metheni M, Lombes A, Bouillaud F, Batteux F, Langsley G. HIF-1alpha induction,
520 proliferation and glycolysis of Theileria-infected leukocytes. *Cell Microbiol.* 2015;17(4):467-
521 72. doi: 10.1111/cmi.12421. PubMed PMID: 25620534.

522 28. Woods KL, Theiler R, Muhlemann M, Segiser A, Huber S, Ansari HR, et al.
523 Recruitment of EB1, a master regulator of microtubule dynamics, to the surface of the
524 *Theileria annulata* schizont. *PLoS Pathog.* 2013;9(5):e1003346. doi:
525 10.1371/journal.ppat.1003346. PubMed PMID: 23675298; PubMed Central PMCID:
526 PMCPMC3649978.

527 29. Zeke A, Bastys T, Alexa A, Garai A, Meszaros B, Kirsch K, et al. Systematic
528 discovery of linear binding motifs targeting an ancient protein interaction surface on MAP
529 kinases. *Mol Syst Biol.* 2015;11(11):837. doi: 10.1525/msb.20156269. PubMed PMID:
530 26538579; PubMed Central PMCID: PMCPMC4670726.

531 30. Wiens O, Xia D, von Schubert C, Wastling JM, Dobbelaere DA, Heussler VT, et al.
532 Cell cycle-dependent phosphorylation of *Theileria annulata* schizont surface proteins. *PLoS*
533 *One.* 2014;9(7):e103821. doi: 10.1371/journal.pone.0103821. PubMed PMID: 25077614;
534 PubMed Central PMCID: PMCPMC4117643.

535 31. Cock-Rada AM, Medjkane S, Janski N, Yousfi N, Perichon M, Chaussepied M, et al.
536 SMYD3 promotes cancer invasion by epigenetic upregulation of the metalloproteinase MMP-
537 9. *Cancer Res.* 2012;72(3):810-20. doi: 10.1158/0008-5472.CAN-11-1052. PubMed PMID:
538 22194464; PubMed Central PMCID: PMCPMC3299564.

539 32. Echebli N, Mhadhbi M, Chaussepied M, Vayssettes C, Di Santo JP, Darghouth MA, et
540 al. Engineering attenuated virulence of a *Theileria annulata*-infected macrophage. *PLoS Negl*
541 *Trop Dis.* 2014;8(11):e3183. doi: 10.1371/journal.pntd.0003183. PubMed PMID: 25375322;
542 PubMed Central PMCID: PMCPMC4222746.

543 33. Zhang Q, Kuang H, Chen C, Yan J, Do-Umehara HC, Liu XY, et al. Corrigendum:
544 The kinase Jnk2 promotes stress-induced mitophagy by targeting the small mitochondrial
545 form of the tumor suppressor ARF for degradation. *Nat Immunol.* 2015;16(7):785. doi:
546 10.1038/ni0715-785b. PubMed PMID: 26086147.

547 34. Marsolier J, Pineau S, Medjkane S, Perichon M, Yin Q, Flemington E, et al. OncomiR
548 addiction is generated by a miR-155 feedback loop in *Theileria*-transformed leukocytes. *PLoS*
549 *Pathog.* 2013;9(4):e1003222. doi: 10.1371/journal.ppat.1003222. PubMed PMID: 23637592;
550 PubMed Central PMCID: PMCPMC3630095.

551 35. Haller D, Mackiewicz M, Gerber S, Beyer D, Kullmann B, Schneider I, et al.
552 Cytoplasmic sequestration of p53 promotes survival in leukocytes transformed by *Theileria*.
553 *Oncogene.* 2010;29(21):3079-86. doi: 10.1038/onc.2010.61. PubMed PMID: 20208567.

554 36. Maggi LB, Jr., Winkeler CL, Miceli AP, Apicelli AJ, Brady SN, Kuchenreuther MJ, et
555 al. ARF tumor suppression in the nucleolus. *Biochim Biophys Acta.* 2014;1842(6):831-9. doi:
556 10.1016/j.bbadi.2014.01.016. PubMed PMID: 24525025.

557 37. Trino S, De Luca L, Laurenzana I, Caivano A, Del Vecchio L, Martinelli G, et al. P53-
558 MDM2 Pathway: Evidences for A New Targeted Therapeutic Approach in B-Acute
559 Lymphoblastic Leukemia. *Front Pharmacol.* 2016;7:491. doi: 10.3389/fphar.2016.00491.
560 PubMed PMID: 28018226; PubMed Central PMCID: PMCPMC5159974.

561 38. Vivo M, Matarese M, Sepe M, Di Martino R, Festa L, Calabro V, et al. MDM2-
562 mediated degradation of p14ARF: a novel mechanism to control ARF levels in cancer cells.
563 *PLoS One.* 2015;10(2):e0117252. doi: 10.1371/journal.pone.0117252. PubMed PMID:
564 25723571; PubMed Central PMCID: PMCPMC4344200.

565 39. Buschmann T, Potapova O, Bar-Shira A, Ivanov VN, Fuchs SY, Henderson S, et al.
566 Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization
567 and transcriptional activities in response to stress. *Mol Cell Biol.* 2001;21(8):2743-54. doi:

568 10.1128/MCB.21.8.2743-2754.2001. PubMed PMID: 11283254; PubMed Central PMCID: 569 PMCPMC86905.

570 40. Heussler VT, Rottenberg S, Schwab R, Kuenzi P, Fernandez PC, McKellar S, et al. 571 Hijacking of host cell IKK signalosomes by the transforming parasite Theileria. *Science*. 572 2002;298(5595):1033-6. doi: 10.1126/science.1075462. PubMed PMID: 12411708.

573 41. Braun L, Brenier-Pinchart MP, Yogavel M, Curt-Varesano A, Curt-Bertini RL, 574 Hussain T, et al. A Toxoplasma dense granule protein, GRA24, modulates the early immune 575 response to infection by promoting a direct and sustained host p38 MAPK activation. *J Exp* 576 *Med.* 2013;210(10):2071-86. doi: 10.1084/jem.20130103. PubMed PMID: 24043761; 577 PubMed Central PMCID: PMCPMC3782045.

578 42. Galley Y, Hagens G, Glaser I, Davis W, Eichhorn M, Dobbelaere D. Jun NH2- 579 terminal kinase is constitutively activated in T cells transformed by the intracellular parasite 580 Theileria parva. *Proc Natl Acad Sci U S A.* 1997;94(10):5119-24. PubMed PMID: 9144200; 581 PubMed Central PMCID: PMCPMC24641.

582 43. Botteron C, Dobbelaere D. AP-1 and ATF-2 are constitutively activated via the JNK 583 pathway in Theileria parva-transformed T-cells. *Biochem Biophys Res Commun.* 584 1998;246(2):418-21. doi: 10.1006/bbrc.1998.8635. PubMed PMID: 9610375.

585 44. Pellegrini E, Palencia A, Braun L, Kapp U, Bougdour A, Belrhali H, et al. Structural 586 Basis for the Subversion of MAP Kinase Signaling by an Intrinsically Disordered Parasite 587 Secreted Agonist. *Structure.* 2017;25(1):16-26. doi: 10.1016/j.str.2016.10.011. PubMed 588 PMID: 27889209; PubMed Central PMCID: PMCPMC5222587.

589 45. Singh S, Khatri N, Manuja A, Sharma RD, Malhotra DV, Nichani AK. Impact of field 590 vaccination with a Theileria annulata schizont cell culture vaccine on the epidemiology of 591 tropical theileriosis. *Vet Parasitol.* 2001;101(2):91-100. PubMed PMID: 11587838.

592 46. Whisenant TC, Ho DT, Benz RW, Rogers JS, Kaake RM, Gordon EA, et al. 593 Computational prediction and experimental verification of new MAP kinase docking sites and 594 substrates including Gli transcription factors. *PLoS Comput Biol.* 2010;6(8). doi: 595 10.1371/journal.pcbi.1000908. PubMed PMID: 20865152; PubMed Central PMCID: 596 PMCPMC2928751.

597 47. Tsirigos KD, Peters C, Shu N, Kall L, Elofsson A. The TOPCONS web server for 598 consensus prediction of membrane protein topology and signal peptides. *Nucleic Acids Res.* 599 2015;43(W1):W401-7. doi: 10.1093/nar/gkv485. PubMed PMID: 25969446; PubMed Central 600 PMCID: PMCPMC4489233.

601 48. Shiels BR, McDougall C, Tait A, Brown CG. Identification of infection-associated 602 antigens in Theileria annulata transformed cells. *Parasite Immunol.* 1986;8(1):69-77. PubMed 603 PMID: 2421227.

604 49. Swan DG, Stadler L, Okan E, Hoffe M, Katzer F, Kinnaird J, et al. TashHN, a 605 Theileria annulata encoded protein transported to the host nucleus displays an association 606 with attenuation of parasite differentiation. *Cell Microbiol.* 2003;5(12):947-56. PubMed 607 PMID: 14641179.

608 50. Narayana N, Cox S, Shaltiel S, Taylor SS, Xuong N. Crystal structure of a 609 polyhistidine-tagged recombinant catalytic subunit of cAMP-dependent protein kinase 610 complexed with the peptide inhibitor PKI(5-24) and adenosine. *Biochemistry.* 611 1997;36(15):4438-48. doi: 10.1021/bi961947+. PubMed PMID: 9109651.

612 **Figures legends:**

613 **Fig 1: JNK2 is predominantly in the infected macrophage cytosol associated with the**
614 **parasite.** Localization of JNK1 (A) and JNK2 (B) in *Theileria*-infected macrophages treated
615 or not with parasiticide drug BW720c. (A) Western blot analysis of nuclear and cytosolic
616 extracts probed with a specific anti-JNK1 antibody and (B) Western blot of nuclear and
617 cytosolic extracts probe with a specific anti-JNK2 antibody. (C) Immunofluorescence image
618 showing association of JNK2 with the parasite decorated by a parasite monoclonal antibody
619 (1C12) to P104. JNK2/P104 co-localization was analysed by the Manders method of pixel
620 intensity correlation measurements using ImageJ/Fiji-Coloc2 plugin, and an average for 30
621 independent cells is given. DNA was stained with DAPI (blue).

622 **Fig 2. p104 interacts with JNK2 in *Theileria*-infected leukocytes.** (A) Immunoprecipitation
623 with a pan anti-JNK (JNK-IP) antibody using whole cell lysates derived from infected (TBL3)
624 and non-infected B cells (BL3), with the precipitate probed with the anti-p104 monoclonal
625 antibody 1C12. **Input**, shows JNK and P104 protein levels in BL3 and TBL3 cells revealed
626 by respective antibodies. (1): BL3; (2): TBL3 1 μ M; (3): IgG; (4): V and (5): input form V.
627 (B) **Right**, Immunoprecipitation with a pan-JNK, specific anti-JNK2, and irrelevant IgG
628 control antibodies with the precipitate from infected macrophages (V) probed with 1C12.
629 **Input**, shows (arrowed) the levels of the p46 JNK1 and p54 JNK2 isoforms revealed with the
630 pan-JNK antibody. An anti-actin antibody was used as a loading control.

631 **Fig 3. Abrogation of JNK2/p104 interaction leads to proteasome-mediated JNK2**
632 **degradation.** Immunoprecipitation analyses with specific anti-JNK2 and anti-JNK1
633 antibodies using whole cell lysates derived from *T. annulata*-infected macrophages treated
634 or not with 1 μ M or 5 μ M of the penetrating JNK-binding motif peptide and treated or not with
635 MG132 (P), mutant (S>A) peptide (mP) or irrelevant peptide. (A) JNK2-IP shows western

636 blot of the JNK2 precipitate probed with the anti-p104, anti-JNK2, and anti-GAPDH
637 antibodies. **Lower panel** shows western blot analysis of immunoprecipitations probed with
638 anti-JNK2 and anti-GAPDH antibodies. (1): V; (2): V+P 1 μ M; (3): V+P 5 μ M; (4): V+P 1 μ M
639 + MG132; (5): V+P 5 μ M+ MG132; (6): V+ mP 1 μ M; (7): V+ mP 5 μ M; (8): IgG. **(B)** JNK1-
640 IP: Immunoprecipitation analyses with anti-JNK1 using whole cell lysates derived from *T.*
641 *annulata*-infected macrophages treated or not with 1 μ M or 5 μ M of P or mP peptides. JNK1
642 protein expression was decreased following the treatment with JNK binding motif competitive
643 peptide, while no effect was observed with the mP peptide. Lower panel shows western blot
644 analysis of immunoprecipitations probed with anti-JNK1 and anti-GAPDH antibodies. (1): V;
645 (2): V+P 1 μ M; (3): V+P 5 μ M; (4): V+mP 1 μ M; (5): V+mP 5 μ M; (6): IgG.

646 Fig 4: **Association with p104 protects JNK2 from ubiquitination and proteosomal
647 degradation. (A)** Immunoprecipitation analyses with anti-JNK2 antibodies using whole cell
648 lysates derived from virulent *T. annulata*-infected macrophages treated or not with 5 μ M of
649 JNK-binding motif peptide P and treated or not with MG132. Western blot of the JNK2
650 precipitate probed with an anti-ubiquitin and JNK2 antibodies. **(B)**. Western blot analysis of
651 nuclear and cytosolic extracts probed with specific anti-JNK1 and anti-JNK2 antibodies using
652 whole cell lysates derived from *T. annulata*-infected macrophages treated or not with 5 μ M of
653 P and treated or not with MG132 and virulent treated with irrelevant peptide. Actin and H3
654 Histone antibodies were used as loading control. Input Panel A, JNK2 levels in the extracts
655 were estimated compared to actin levels were used as loadingcontrol. Panel B, JNK1 and
656 JNK2 levels in nuclear extracts were compared to histone H3 levels.

657 **Fig 5. PKA phosphorylation increases association of p104 with JNK2. (A)**
658 Immunoprecipitation analyses with an anti-JNK2 antibody using whole cell lysates derived
659 from virulent *T. annulata*-infected macrophages treated or not with the PKA specific inhibitor
660 myristoylated PKI (MyrPKI) and H89. Left panel shows western blot of the JNK2 precipitate

661 from non-treated, or MyrPKI /H89 treated (+) cells probed with the anti-p104 1C12
662 monoclonal antibody. Middle and right panels show the input levels of p104 and actin
663 revealed by their respective antibodies. **(B)** Left panel: Western blot of JNK2 precipitates
664 using extracts of cells treated with a pan-JNK inhibitor (pan-JNKi), or a JNK2-specific
665 inhibitor (JNK2i) probed with the anti-p104 1C12 monoclonal antibody. Right panel: shows
666 the input levels of p104 and the two JNK isoforms revealed by their respective antibodies.

667 **Fig 6. Peptide-provoked disruption of the JNK2/p104 complex diminishes matrigel**
668 **traversal of *T. annulata*-transformed macrophages.** **(A)** Upper panel: Matrigel traversal of
669 virulent (V) compared to attenuated (A) macrophages and virulent macrophages treated with
670 the JNK-motif penetrating peptide, the mutant (S>A) peptide (mP), or control irrelevant
671 peptides (irrP). Peptide (P)-provoked disruption of the JNK2/p104 complex reduced matrigel
672 traversal of virulent macrophages (V+P) to below attenuated levels, whereas treatment of
673 virulent macrophages with the mutant (S>A) peptide (V+mP), or control peptide (irrP) had no
674 effect. **(B) Left panel.** Zymogram showing MMP9 activity in the supernatants of virulent (V)
675 compared to attenuated (A) macrophages and virulent macrophages treated with the
676 competitive JNK2-binding peptide (P). **Right panel.** Relative expression of *mmp9* in virulent
677 and attenuated *Theileria*-infected treated or not with the competitive JNK-binding peptide (P),
678 or the mutant (S>A) peptide (mP). **(C) Upper panel.** Nuclear c-Jun phosphorylation
679 displayed by virulent macrophages treated or not with the competitive JNK-binding peptide
680 (P), the mutant (S>A) peptide (mP) or control peptides (irrP). Scale bar is equivalent to 10 μ
681 meters. **Bottom panel.** Percentage of corrected total cell fluorescence due to phospho-Ser73-
682 c-Jun staining based on 30-independent cell images. All experiments were done
683 independently (n = 3). The error bars show SEM values from 3 biological replicates, * p value
684 < 0.05, *** p value < 0.001.

685 **Fig 7. Loss of JNK2 provokes appearance of smARF and induction of autophagy. (A)**

686 Loss of JNK2 provoked by treating virulent macrophages (lane 1) with 1 μ M (lane 2) and
687 5 μ M (lane 3) of the penetrating JNK-binding motif peptide causes a dose-dependent increase
688 in the amounts of p14 ARF. No effect was observed with 5 μ M of mutant peptide (lane 4).
689 **Bottom**, virulent macrophages (lane 1) were treated or not with 5 μ M of penetrating JNK-
690 binding motif peptide (lane 2) and cell extracts probed with the specific LC3B-II antibody.
691 5 μ M peptide treatment results in augmented amounts of processed LC3B-II. No effect was
692 observed with 5 μ M of mP (lane 3). **(B)**. Immuno fluorescence images obtained with anti-LC3B-
693 II antibody using virulent (V) macrophages treated or not with JNK-binding motif peptide (P),
694 or mutant peptide (mP). Only in peptide treated (V+P) macrophages is an augmentation in
695 LC3B-II and clustering of LC3B-II-positive structures evident. No fluorescence was observed
696 with Alexa-labelled secondary antibody (Vc). Scale bar is equivalent to 10 μ meters. **Bottom**.
697 Percentage of corrected total cell fluorescence due to LC3B-II staining based on 25-
698 independent cell images. All experiments were done independently (n = 3). The error bars
699 show SEM values from 3 biological replicates ** p value < 0.01.

700 **Supporting information:**

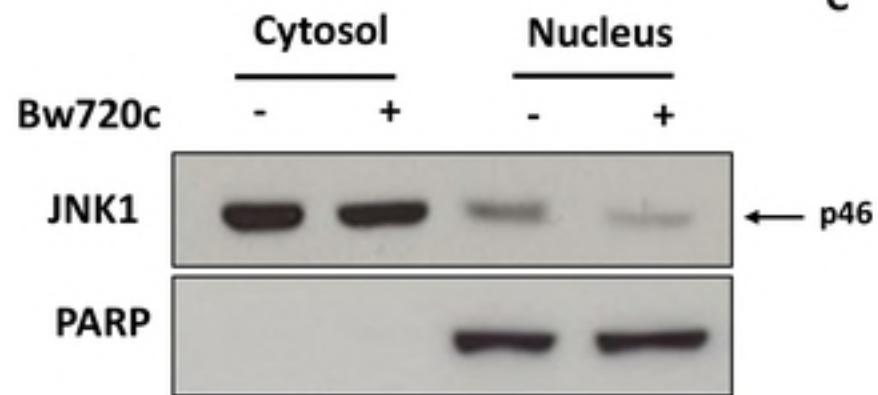
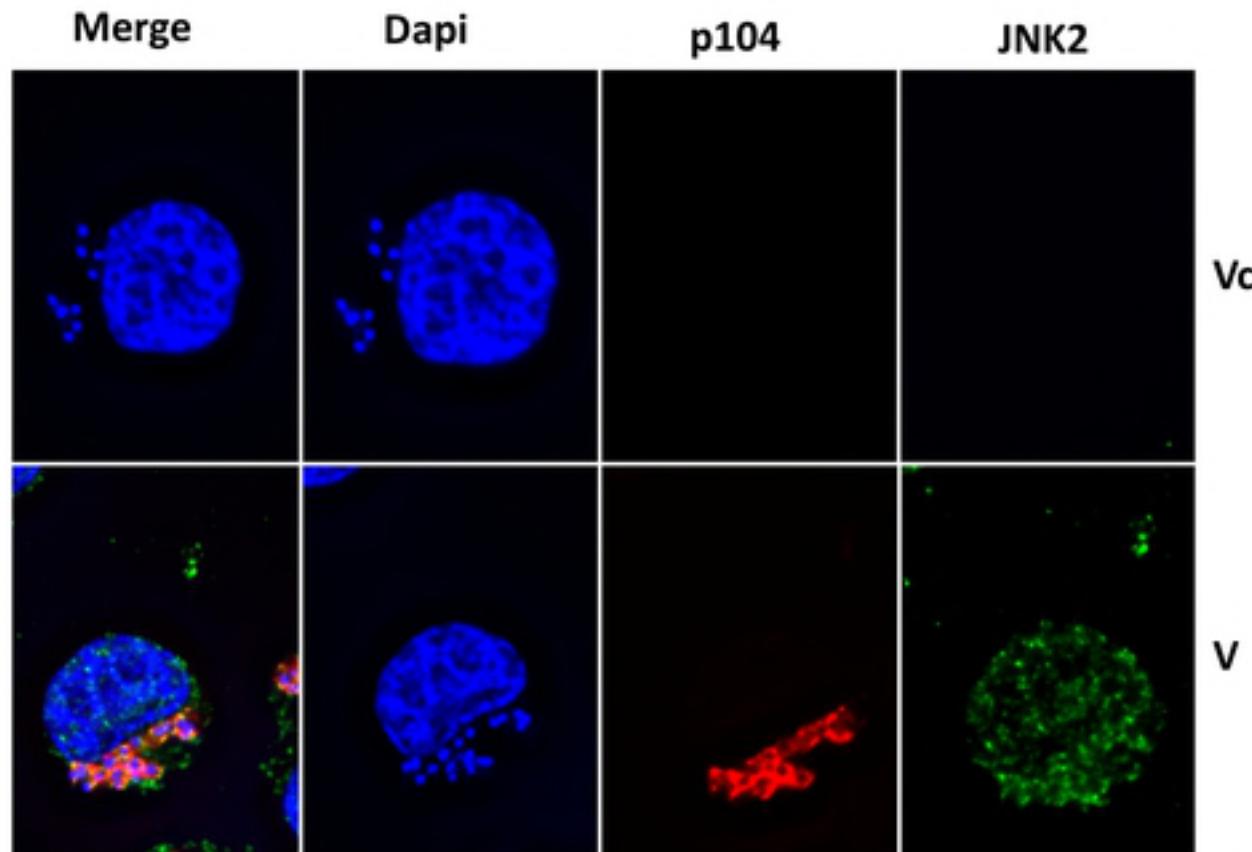
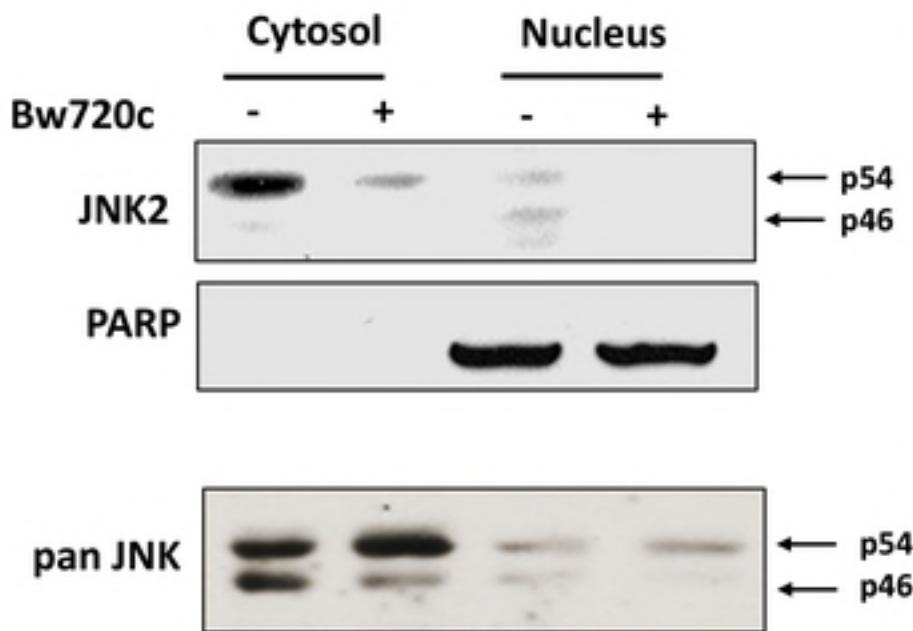
701 **S1 file:** Screening for potential JNK binding sites (D-site) using dfinder with a 1e-23 cutoff
702 and the predicted proteomes of *Theileria annulata*, *T. parva* and *T. orientalis*.

703 **S1 Fig: Penetrating peptides enter *T. annulata*-infected macrophages.** Virulent *T.*
704 *annulata*-infected macrophages are treated with FITC-conjugated peptide for 2h. Green:
705 peptide; Dapi: nucleus.

706 **S2 Fig: (A) GST pulldown with JNK2 and p104 recombinant proteins.** Recombinant
707 proteins were expressed in *E. coli* and purified. Baits (lane 1, GST+ JNK2, Lane 2, p104,
708 Lane 3, JNK2) were loaded to glutathione resin and were incubated with JNK2 (Load, Lane 4).

709 Samples were run on SDS-PAGE and the gel was stained with Coomassie dye, or was
710 subjected to Western blot and protein bands visualized by an anti-His antibody (as all purified
711 proteins had a 6xHis tag). (M – Marker, molecular weights are shown in kDa at the left). **(B)**
712 ***In vitro* PKA-mediated phosphorylation of recombinant p104.** 0.5 μ M of the PKA
713 catalytic subunit was incubated with 5 μ M GST or GST-p104 fusion proteins in the presence
714 of radioactively labelled ATP(γ P)32. Aliquots of the kinase reactions were taken at the
715 indicated time points and run on SDS-PAGE. The upper panel shows the gel stained with
716 Coomassie dye and the lower panel shows the phosphor imaging results of the same gel. (M –
717 Marker, molecular weights are shown in kDa at the left).

718 **S3 Figure : 24 h after peptide-induced disruption of JNK2/p104 complex *Theileria*-**
719 **infected macrophages become Annexin V-positive.** Infected macrophages were treated for
720 24 h with 1 μ M or 5 μ M of JNK-motif penetrating peptide or the mutant (S>A) peptide (mP)
721 in presence or absence of MG132 and the percentage of cells undergoing apoptosis was
722 estimated by FACS using annexin V/ 7AAD staining.

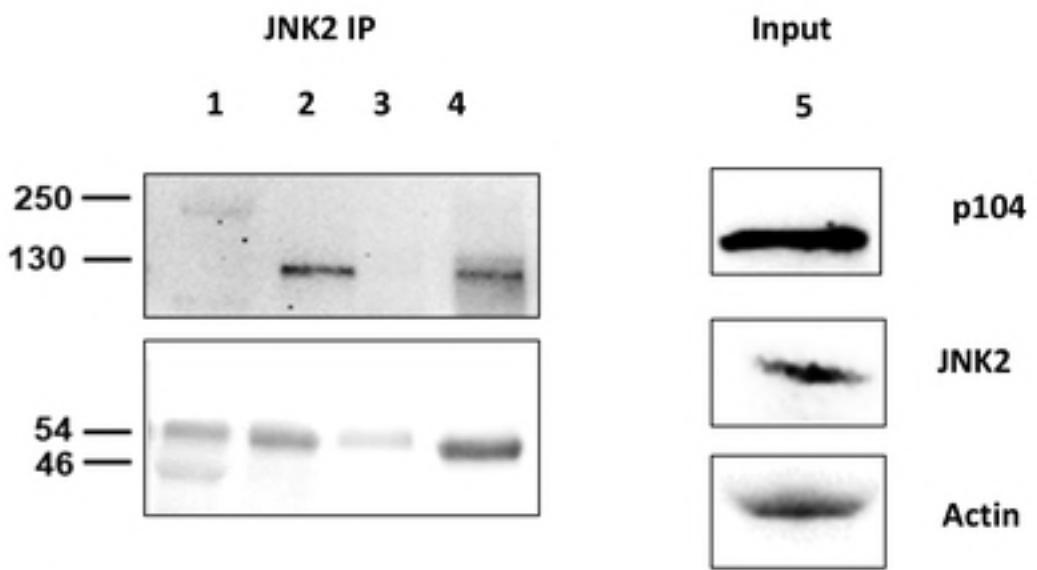
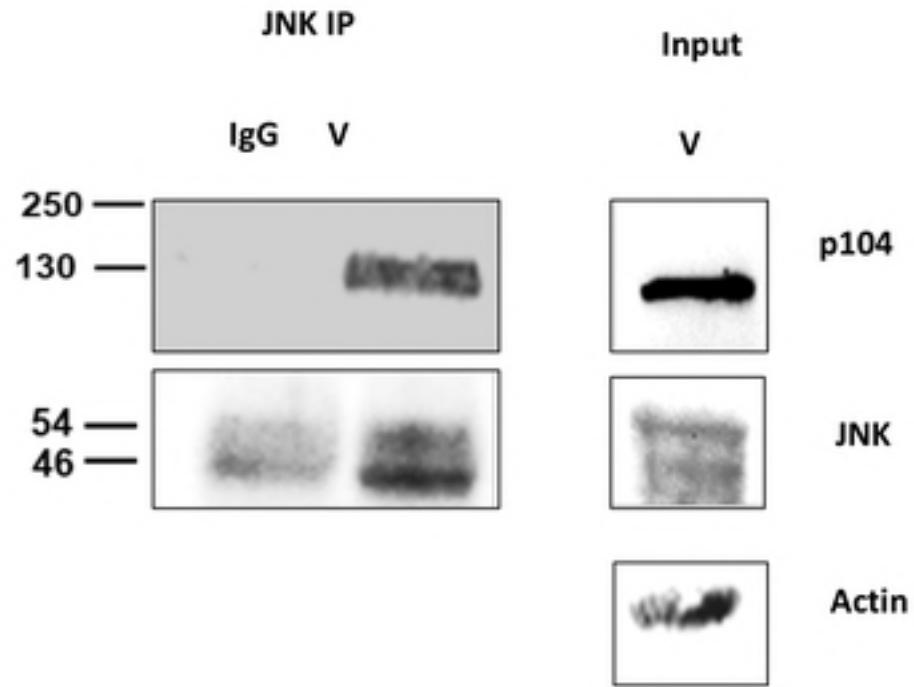
A**C****B**

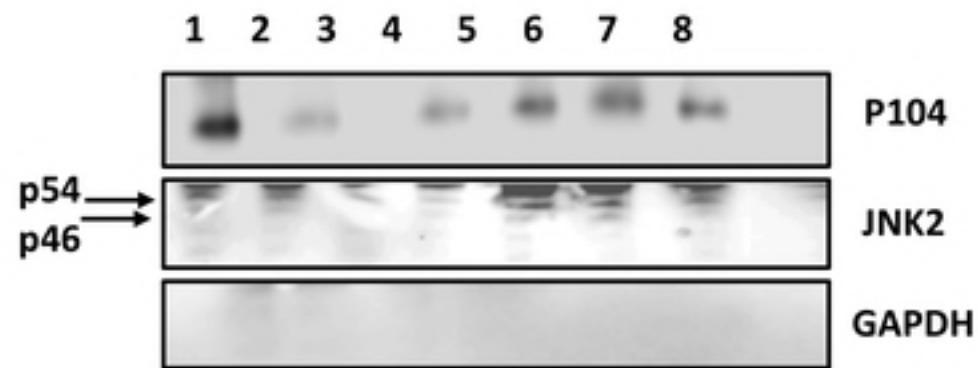
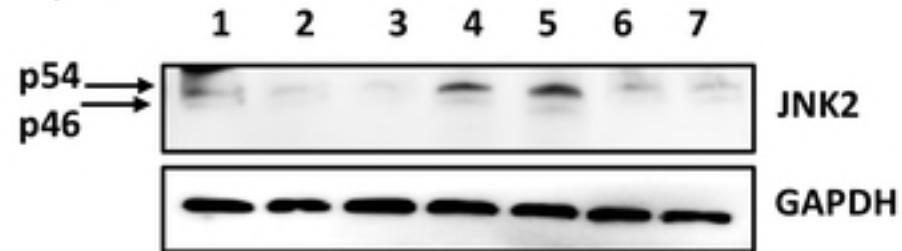
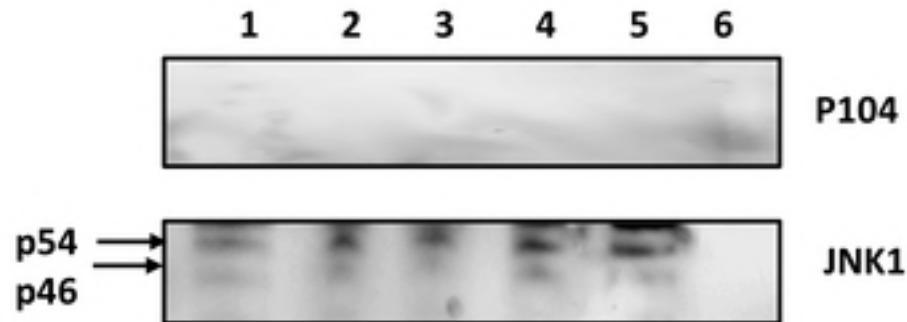
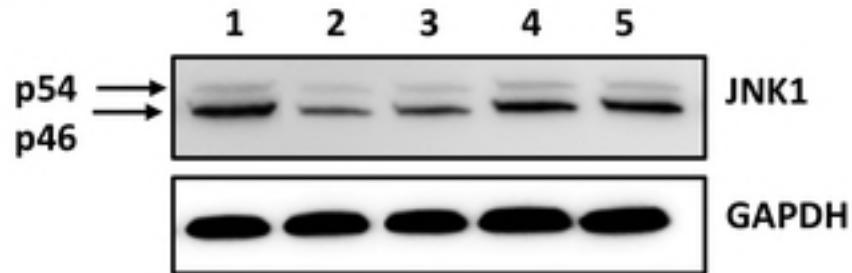
N=30

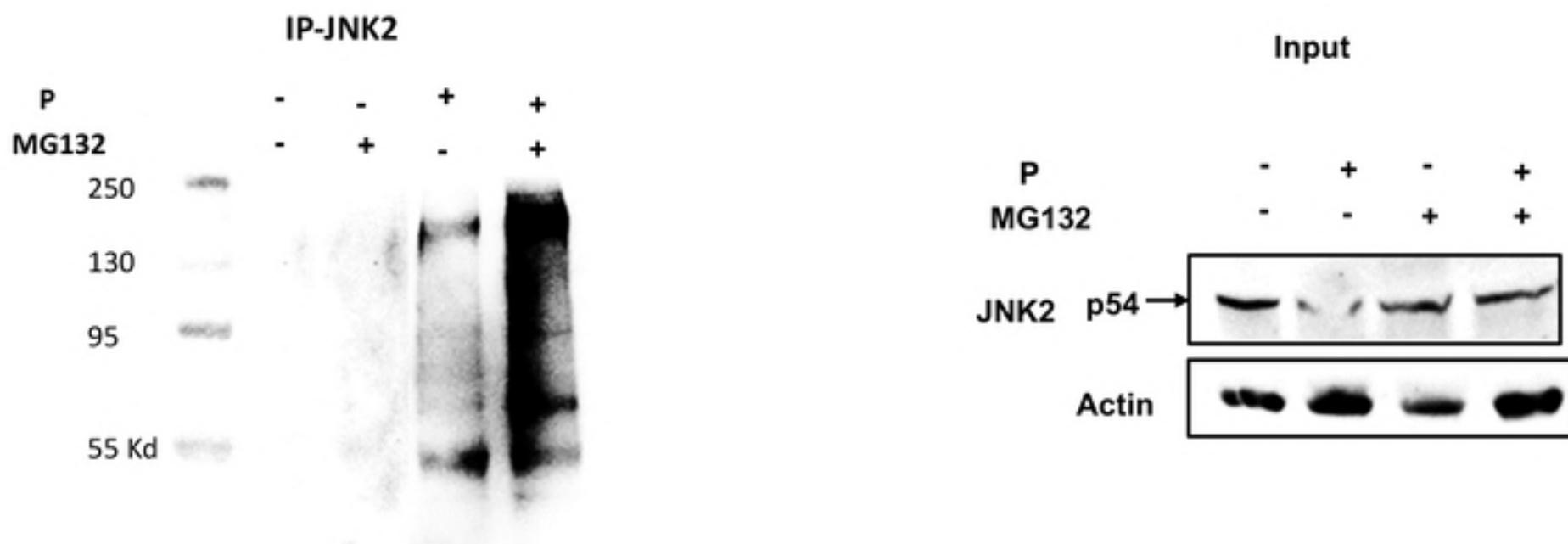
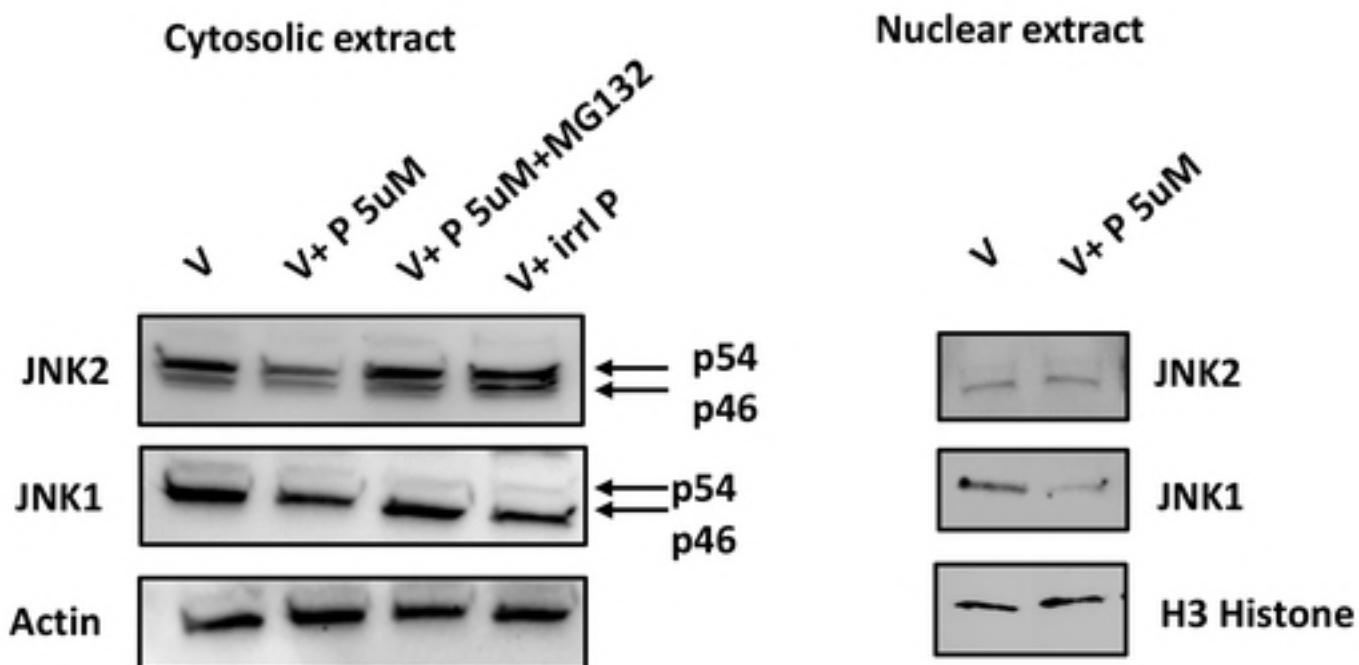
Pearson's coefficient 0.78

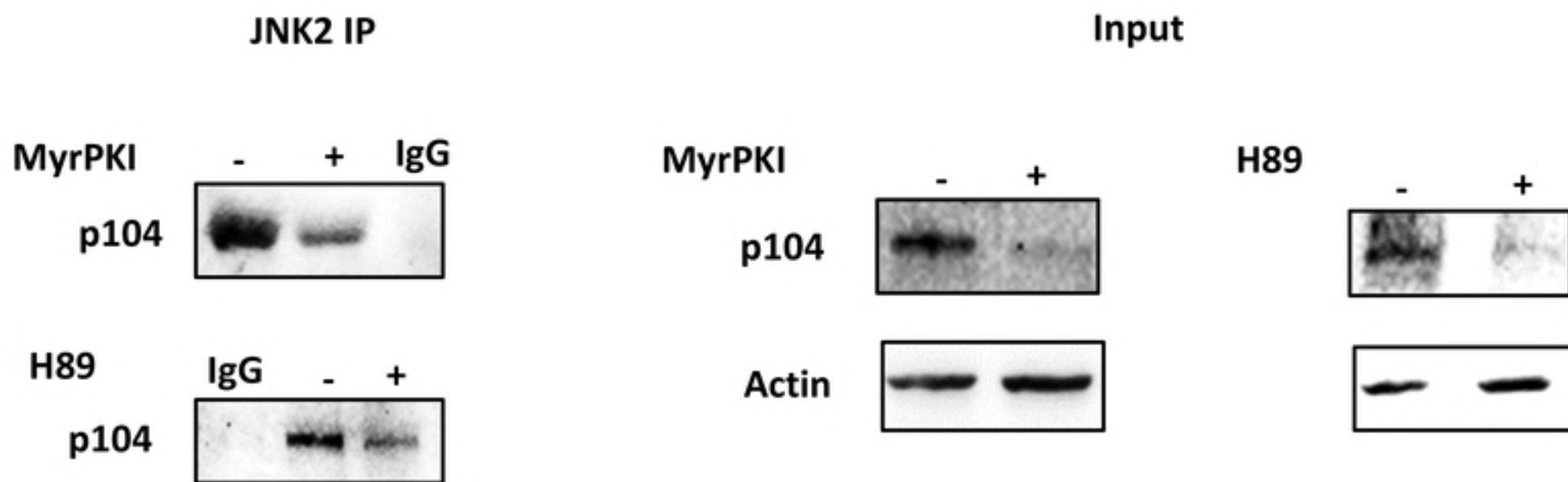
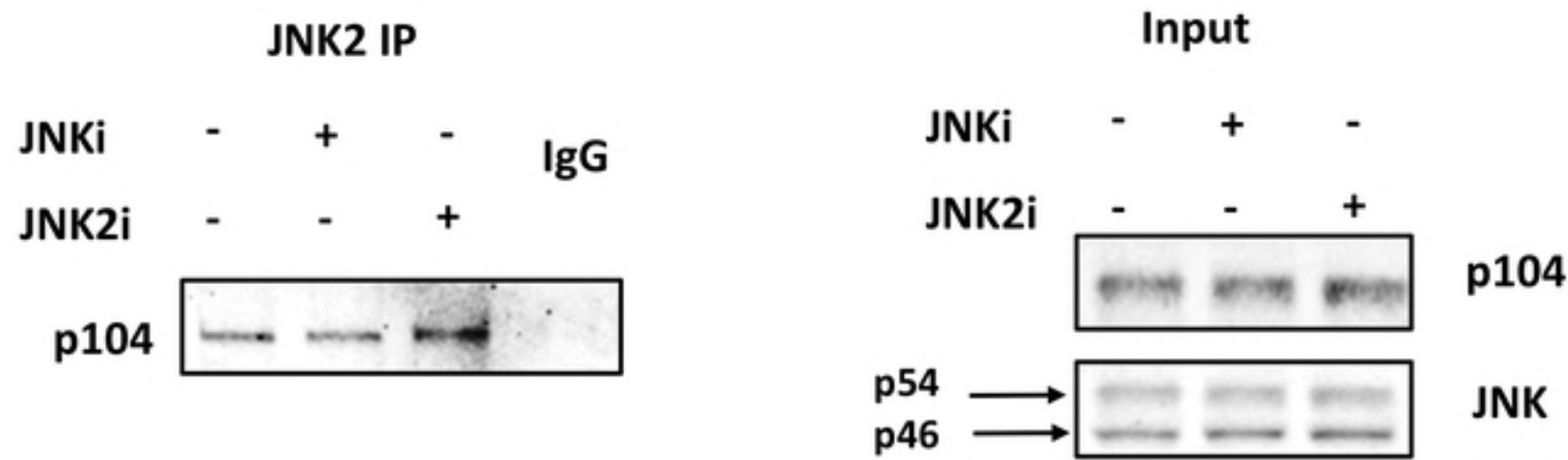
Vc

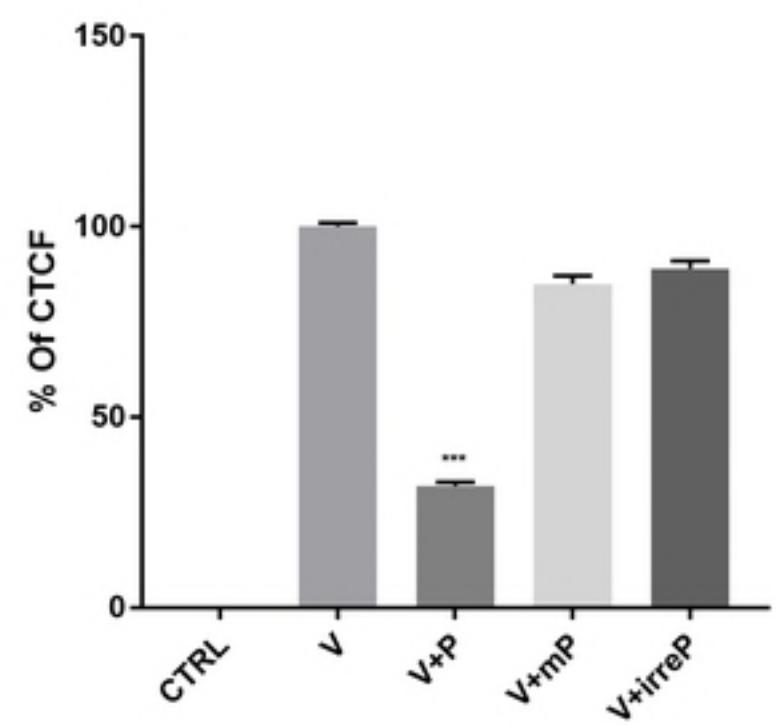
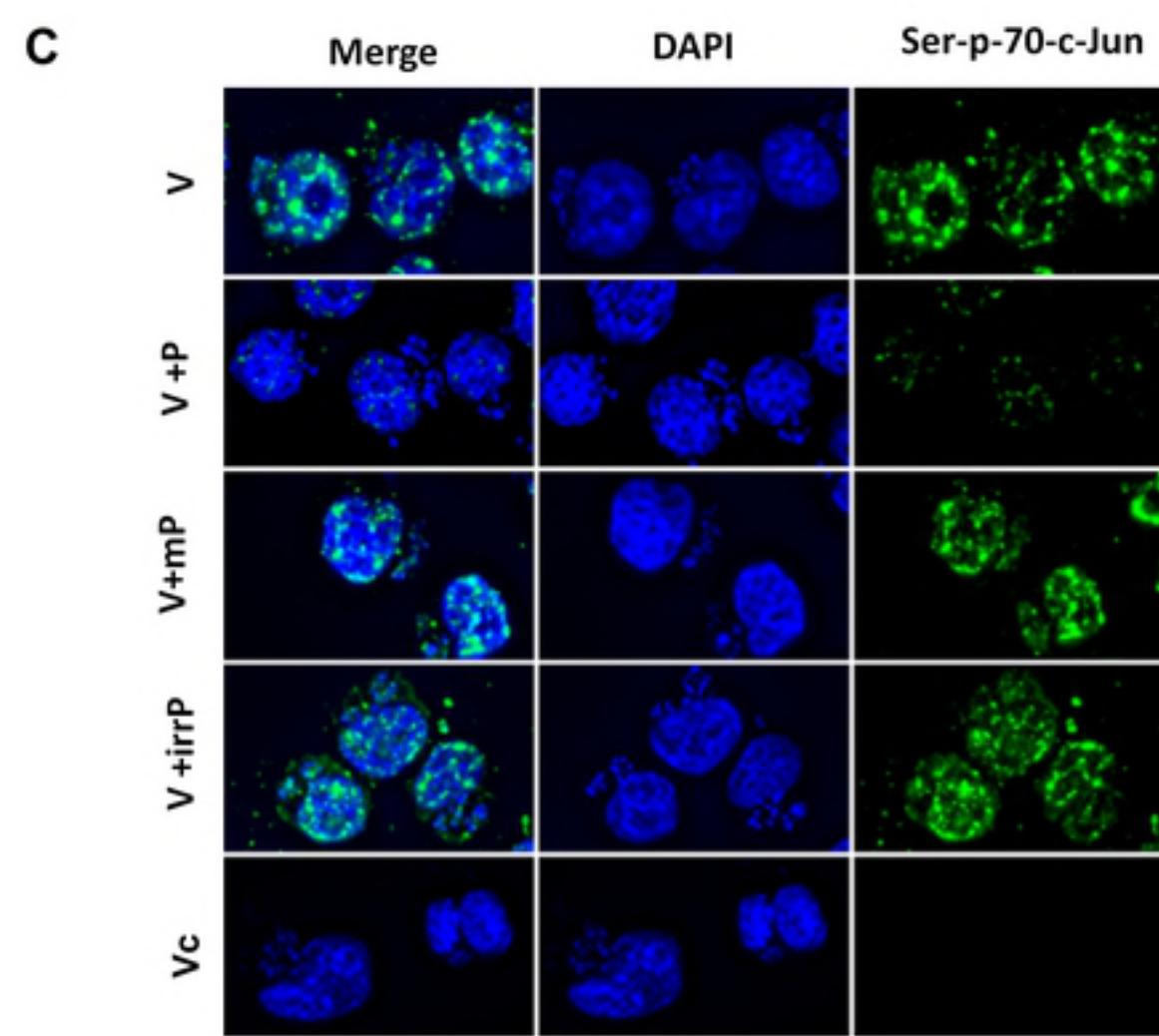
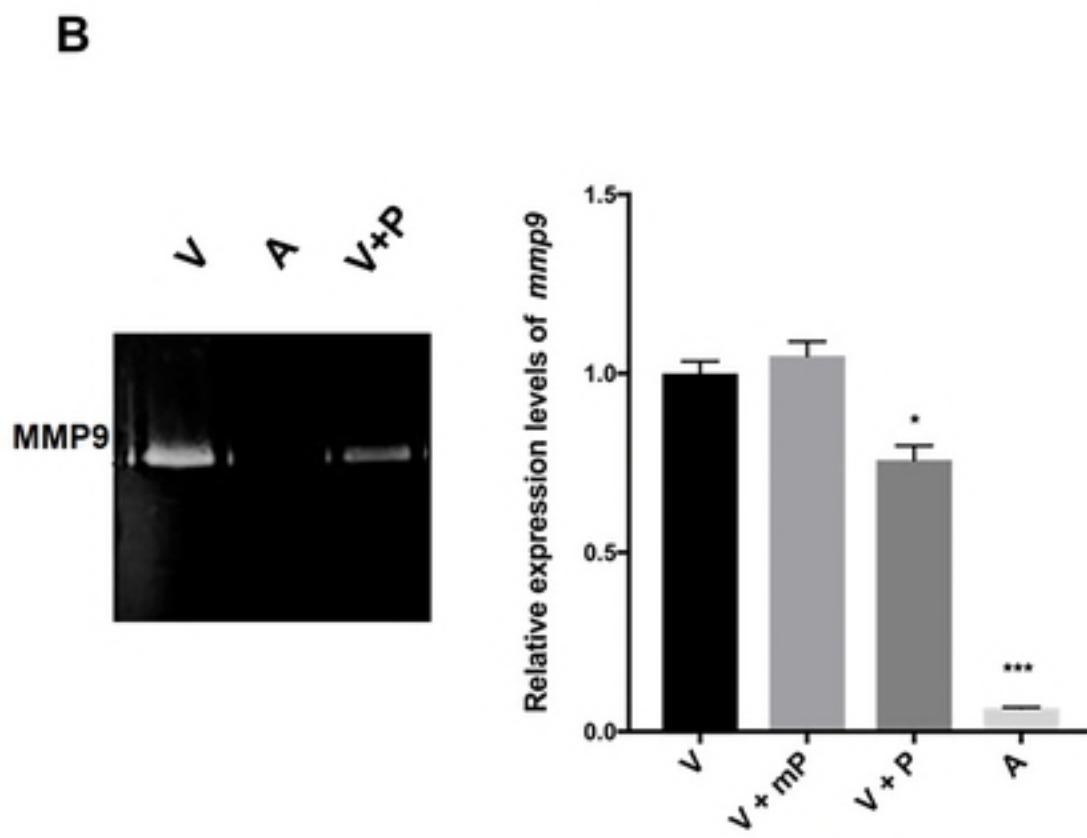
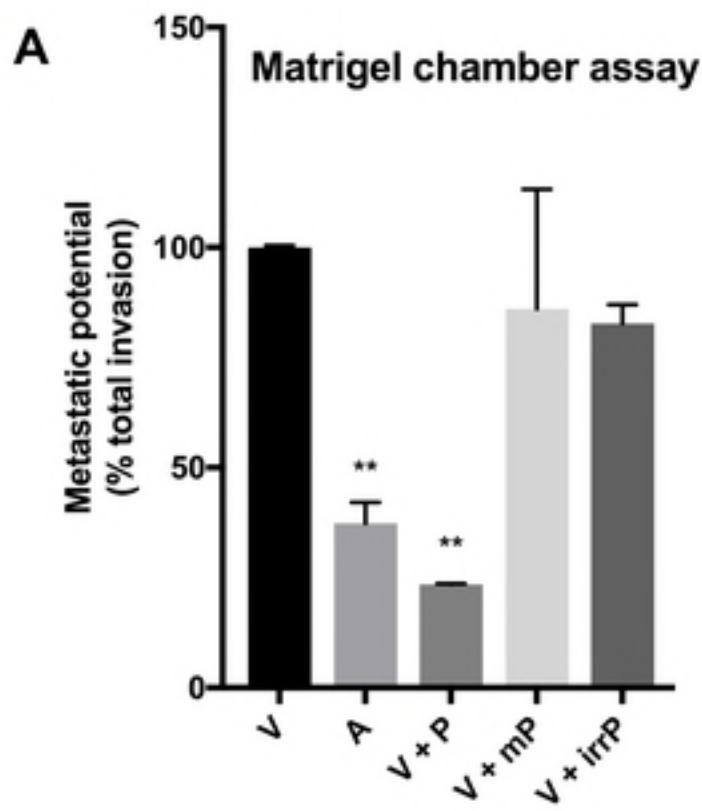
V

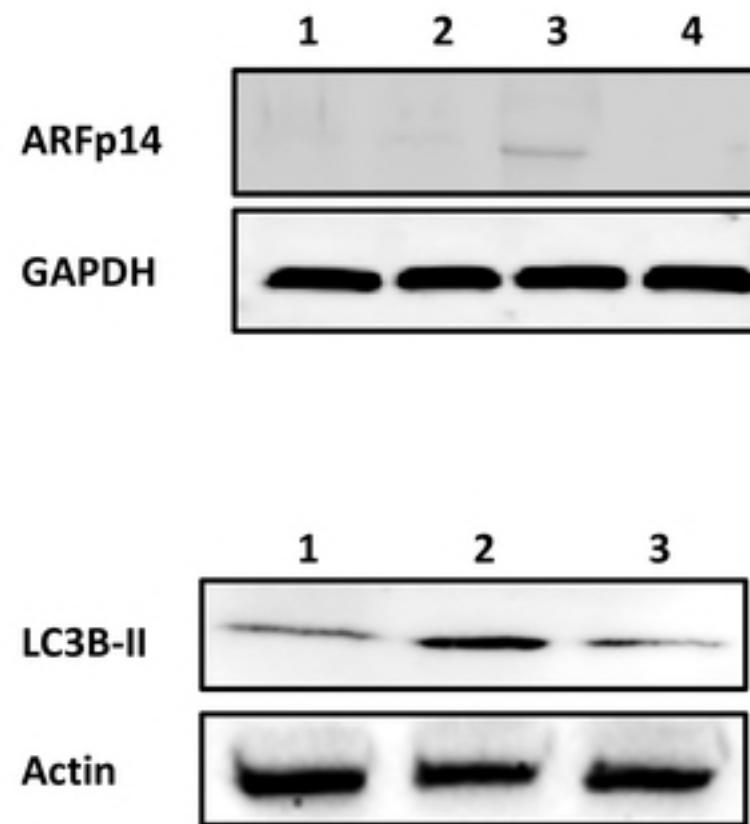
A**B**

A**IP-JNK2****Input****B****IP-JNK1****Input**

A**B**

A**B**



A**B**