1   **Biogenesis of P-TEFb in CD4^{+} T cells to reverse HIV latency is mediated by protein**

2   **kinase C (PKC)-independent signaling pathways**

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Abstract

The switch between HIV latency and productive transcription is regulated by an auto-feedback mechanism initiated by the viral trans-activator Tat, which functions to recruit the host transcription elongation factor P-TEFb to proviral HIV. A heterodimeric complex of CDK9 and one of three cyclin T subunits, P-TEFb is expressed at vanishingly low levels in resting memory CD4$^+$ T cells and cellular mechanisms controlling its availability are central to regulation of the emergence of HIV from latency. Using a well-characterized primary T-cell model of HIV latency alongside healthy donor memory CD4$^+$ T cells, we characterized specific T-cell receptor (TCR) signaling pathways that regulate the generation of transcriptionally active P-TEFb, defined as the coordinate expression of cyclin T1 and phospho-Ser175 CDK9. Protein kinase C (PKC) agonists, such as ingenol and prostratin, stimulated active P-TEFb expression and reactivated latent HIV with minimal cytotoxicity, even in the absence of intracellular calcium mobilization with an ionophore. Unexpectedly, inhibition-based experiments demonstrated that PKC agonists and TCR-mobilized diacylglycerol signal through MAP kinases ERK1/2 rather than through PKC to effect the reactivation of both P-TEFb and latent HIV. Single-cell and bulk RNA-seq analyses revealed that of the four known isoforms of the Ras guanine nucleotide exchange factor RasGRP, RasGRP1 is by far the predominantly expressed diacylglycerol-dependent isoform in CD4$^+$ T cells. RasGRP1 should therefore mediate the activation of ERK1/2 via Ras-Raf signaling upon TCR co-stimulation or PKC agonist challenge. Combined inhibition of the PI3K-mTORC2-AKT-mTORC1 pathway and the ERK1/2 activator MEK prior to TCR co-stimulation abrogated active P-TEFb expression and substantially suppressed latent HIV reactivation. Therefore, contrary to prevailing models, the coordinate reactivation of P-TEFb and latent HIV in primary T cells following either TCR co-stimulation or PKC agonist challenge is independent of PKC but rather involves two complementary signaling arms of the TCR cascade, namely, RasGRP1-Ras-Raf-MEK-ERK1/2 and PI3K-mTORC2-AKT-mTORC1.

49 **Author Summary**

50 Dissecting the cellular pathways through which HIV emerges from latency is a key step in the

51 development of therapeutically viable approaches for latency reversal and eventual clearance of

52 persistent HIV in infected individuals. The essential host transcription elongation factor P-TEFb,

53 a heterodimer of CDK9 kinase and a regulatory cyclin T subunit, is a critical mediator of the trans-

54 activation of latent HIV. Availability of P-TEFb for proviral transcription is highly limited due to a

55 posttranscriptional restriction in cyclin T1 expression and dephosphorylation of CDK9 on its

56 activation loop. Using a well-characterized primary T-cell model of HIV latency alongside healthy

57 donor memory CD4$^+$ T cells, we have now defined the signaling pathways that are essential for

58 the generation of transcriptionally active P-TEFb and, consequently, proviral reactivation. Crucial

59 among these findings is the demonstration that protein kinase C (PKC) agonists signal through

60 off-target activation of RasGRP1-Ras-Raf-MEK-ERK1/2 to effect the reactivation of both P-TEFb

61 and proviral HIV. Understanding these pathways should lead to the discovery of novel highly

62 selective activators of P-TEFb to improve the efficiency of HIV reactivation in the memory T-cell

63 population of virally suppressed individuals.

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Introduction

Comprehensively understanding the host mechanisms that permit HIV to emerge from transcriptional latency is crucial for the development of safe pharmacological strategies to eliminate persistent viral reservoirs that are primarily found within memory CD4^{+} T cells of infected individuals [1-5]. The switch between HIV latency and productive transcription is regulated by an auto-feedback mechanism initiated by the viral trans-activator protein Tat, which functions to recruit the essential host transcription elongation factor P-TEFb along with the super elongation complex (SEC) to proviral HIV [6-9]. P-TEFb is a heterodimer of CDK9 serine/threonine kinase and one of three cyclin T regulatory subunits. However, cyclin T1 (CycT1) is the only cyclin capable of interacting with HIV Tat [7, 10, 11].

Because of Tat's auto-feedback mechanism, the transcriptional inactivation of HIV to achieve latency occurs in the absence of Tat expression as acutely infected T cells revert to a resting memory phenotype with the consequent restriction of the nuclear activity of P-TEFb [12, 13]. Subsequently, this results in formation of epigenetic structures at the fixed nucleosome around the viral promoter region (Nuc-1) which hinder the efficient recruitment of RNA polymerase II (RNAP II). The functional importance of these repressive epigenetic modifications is clearly evidenced by the observations that small molecule compounds that inhibit histone deacetylation or methylation can reactivate transcriptionally silenced HIV in cell line and primary cell models of HIV latency [14, 15]. However, even though histone deacetylase inhibitors are thus far the only class of latency reversal agents (LRAs) to achieve clinical proof-of-concept, they have been found to be marginally effective at activating latent proviruses in both primary cell models of HIV latency and patient-derived CD4^{+} T cells [16-22]. In a recent clinical study of the HDACi romidepsin we found that poor HIV reactivation was associated with its limited ability to reactivate P-TEFb and transcription initiation factors [23].

94   Maintenance of a transcriptionally inactive state in resting memory CD4$^+$ T cells, which

95   engenders the persistence of proviral HIV in latently infected cells, is associated with a tight

96   restriction imposed on P-TEFb activity [24, 25]. T-cell receptor (TCR) activation of primary CD4$^+$

97   T cells potently provides the intracellular signals that are necessary to efficiently stimulate

98   processive HIV transcription by reversing the epigenetic silencing at the proviral promoter,

99   inducing the assembly of P-TEFb and permitting nuclear entry of transcription initiation factors

100   [12, 13]. Therefore, the development of more efficient latency reversal strategies will likely require

101   a combination of agents that mimic the effects of TCR signaling.

102   Physiological TCR signaling in CD4$^+$ T cells occurs in the context of an antigen presenting

103   cell (APC) that has on its surface pathogen-derived peptides bound to MHC Class II

104   transmembrane proteins and involves simultaneous activation of multiple signaling pathways. For

105   maximal T-cell activation to be enabled, binding of the peptide-MHC complex to the TCR is

106   accompanied by a costimulatory engagement by the APC that usually involves a pairwise linkage

107   between B7 and its T-cell counterpart CD28 [26]. TCR co-stimulation immediately induces a

108   tyrosine phosphorylation cascade at the cell membrane that leads to the activation of the lipid

109   metabolizing enzymes phospholipase C-γ (PLC-γ) and phosphoinositide-3-kinase (PI3K) [27]. In

110   turn, PLC-γ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to generate the second

111   messengers inositol-1,4,5-triphosphate (IP$_3$), that mobilizes calcium release into the cytoplasm,

112   and diacylglycerol (DAG), which directly contributes to the activation of a number of C1 domain-

113   containing intracellular signaling proteins including protein kinase C (PKC) enzymes and the Ras

114   guanine nucleotide exchange factor RasGRP [28-30]. Class I PI3K enzymes are directly bound

115   to CD28 at the T-cell plasma membrane via a well-characterized YMNM motif on the cytoplasmic

116   domain of the costimulatory receptor [31-33]. Upon their activation, PI3Ks act to phosphorylate

117   PIP$_2$ to form phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$), a lipid resistant to PLC-γ hydrolysis,

118   that serves to anchor a wide range of signaling proteins through their pleckstrin homology (PH)

119   domains [34].

120   Biochemical experiments carried out using both Jurkat T-cell and primary cell models of

121   HIV latency [35-39], showed that the activation of latently infected resting primary CD4$^+$ T cells

122   upon TCR engagement triggers the rapid nuclear induction of transcription initiation factors such

123   as NFAT, AP-1 and NF-κB. Once in the nucleus these factors bind the proviral promoter and

124   initiate transcription by recruiting histone modifying and chromatin remodeling enzymes whose

125   activity on chromatin at or around the transcription start site allows for formation of the preinitiation

126   complex (PIC) and promoter recruitment of RNAP II.

127   In contrast to Jurkat T cells, primary resting CD4$^+$ T cells are also highly deficient in the

128   expression of the CycT1 subunit of P-TEFb while the CDK9 kinase subunit is sequestered in an

129   inactive form in the cytoplasm bound to the kinase-specific chaperone complex Hsp90/Cdc37 [24,

130   25]. P-TEFb has been found to be restricted in resting T cells by a combination of microRNA-

131   mediated repression of CycT1 protein synthesis and dephosphorylation of CDK9 on its regulatory

132   activation loop (T-loop) at the highly conserved residue Thr186 [40, 41]. Activation of resting T

133   cells stimulates a rapid posttranscriptional synthesis of CycT1, which is tightly coupled with

134   Thr186 phosphorylation of the CDK9 subunit (pThr186 CDK9), a posttranslational modification

135   that is critical for the stable heterodimeric assembly of P-TEFb [25]. Once P-TEFb is assembled

136   it is incorporated into 7SK snRNP, a nuclear regulatory ribonucleoprotein complex that serves to

137   inhibit its kinase activity and sequester the enzyme in nucleoplasmic substructures called

138   speckles found in close proximity to genomic sites of active transcription [42-45]. Recruitment of

139   P-TEFb from 7SK snRNP by Tat and their cooperative binding to a nascently synthesized RNA

140   hairpin called TAR puts the enzyme in close proximity to the negative elongation factors NELF

141   and DSIF, and promoter-proximally paused RNAP II. CDK9 then acts to phosphorylate the E

142   subunit of the repressive NELF complex at multiple serine residues which causes NELF to

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143   dissociate from TAR [46-48]. CDK9 also extensively phosphorylates the CTD of RNAP II mainly

144   at Ser2 residues of the heptad repeats Y-S-P-T-S-P-S and C-terminal repeats (G-S-Q/R-T-P) of

145   the hSpt5 subunit of DSIF at Thr4 residues [49-51]. The overall effect of these phospho-

146   modifications by P-TEFb is to remove the blocks to elongation imposed by NELF and DSIF and

147   to stimulate efficient elongation and co-transcriptional processing of proviral transcripts.

148   7SK snRNP comprises a molecule of 7SK snRNA that is bound at its 5' and 3' ends by

149   the capping enzyme MEPCE and the La-related protein LARP7, respectively [52, 53]. The three-

150   dimensional folding conformation of 7SK snRNA establishes a molecular scaffold that

151   cooperatively binds two molecules of P-TEFb to a homodimer of the inhibitory protein HEXIM1

152   [54, 55]. Incorporation of P-TEFb into 7SK snRNP requires both Thr186 phosphorylation of CDK9

153   and the expression of HEXIM1 [25, 56-58], both of which have restricted expression in resting

154   memory CD4^{+} T cells [24]. In cells that are actively dividing, it's estimated that at least half of all

155   cellular P-TEFb is found in 7SK snRNP [59], clearly denoting an important role this

156   ribonucleoprotein complex plays in regulating global transcription elongation. In a proteomics

157   study designed to identify posttranslational modifications on HEXIM1 that regulate the

158   sequestration of P-TEFb by 7SK snRNP, we found that HEXIM1-associated 7SK snRNP can also

159   serve to retain P-TEFb in the nucleus most likely to provide an exchangeable pool of the enzyme

160   that can be readily availed to stimulate gene transcription [57]. An additional CDK9 T-loop

161   phospho-modification occurs at a conserved residue Ser175 (pSer175 CDK9) which we recently

162   demonstrated is due to CDK7 activity in both Jurkat and primary CD4^{+} T cells [25]. pSer175 CDK9

163   is only found on a subset of P-TEFb that is transcriptionally active (ie. P-TEFb dissociated from

164   7SK snRNP) and is functionally important in being coopted by Tat to enhance Tat's interaction

165   with P-TEFb in order to outcompete BRD4 [25, 60], a bromodomain-containing protein that is

166   considered to be a major recruiter of P-TEFb to cellular genes [59, 61].

167   Our current study focuses on defining signaling pathways and T-cell factors that are

168   responsible for regulating proviral reactivation in memory T cells, with a specific focus on

169   identifying the pathways that are essential for the generation of transcriptionally active P-TEFb.

170   PKC agonists are well known and highly potent HIV latency reversing agents, but although

171   progress has been made with development of novel synthetic agonists, they have had limited

172   clinical applications due to concerns about their side effects and cytotoxicity [62, 63]. Using a well-

173   characterized and highly reproducible primary QUECEL (quiescent effector cell latency) model of

174   HIV latency [64], alongside healthy donor memory CD4$^+$ T cells, we found that diacylglycerol-

175   mimicking PKC agonists can stimulate active P-TEFb expression with minimal cytotoxicity in the

176   absence of co-treatment with a calcium ionophore. Unexpectedly, PKC agonists induce P-TEFb

177   via off-target activation of RasGRP1-Ras-Raf-MEK-ERK1/2. P-TEFb induction after TCR co-

178   stimulation is further regulated by the PI3K-mTORC2-AKT-mTORC1 pathway. Combined

179   inhibition of the PI3K-mTORC2-AKT-mTORC1 pathway and the ERK1/2 activator MEK prior to

180   TCR co-stimulation completely abrogated active P-TEFb expression and substantially

181   suppressed latent HIV reactivation. Thus, reactivation of P-TEFb in primary T cells in response to

182   either TCR co-stimulation or PKC agonist challenge is largely independent of PKC suggesting

183   novel opportunities to develop effective latency reversing strategies.

184   **Results**

185   **Posttranscriptional induction of P-TEFb expression in primary T cells is required**

186   **for latent proviral reactivation**

187   Resting CD4$^+$ T cells serve as the primary reservoir for latent HIV and maintain a cellular

188   environment that is non-permissive for HIV transcription. Unstimulated memory CD4$^+$ T cells

189   isolated from healthy donor blood (**S1A Fig**), the majority of which possess a central memory

190   phenotype (**S1B Fig**), are largely in a resting or quiescent state. Characteristically, the resting

191   cells lack markers of T-cell activation (CD25 and CD69), cell proliferation (Ki67 and cyclin D3)

192   and critical transcription factors such as NF-κB and P-TEFb (**S2 and S3 Figs**).

193   As shown in **Fig 1A,** P-TEFb activity is highly restricted in resting memory CD4$^+$ T cells

194   due to a posttranscriptional block in CycT1 translation and sequestration of activation-loop-

195   dephosphorylated CDK9 in the cytoplasm by Hsp90/Cdc37 [25, 40, 41]. We have developed

196   sensitive flow cytometry assays to monitor CycT1 expression and phosphorylation of CDK9 on its

197   activation loop (T-loop) after stimulation of memory CD4$^+$ T cells (**Figs 1B and 1C**) [25, 60]. Using

198   this assay with a range of known latency reversing agents revealed that T-cell receptor (TCR) co-

199   stimulation with anti-CD3 and anti-CD28 coated beads is the most effective at eliciting induction

200   of CycT1 expression and T-loop phosphorylation of CDK9 in memory CD4$^+$ T cells (**Fig 1D**).

201   Activation of memory T cells through the TCR results in a rapid synthesis of CycT1 protein (**Fig**

202   **1B and S3 Fig**) and, just as rapid, the localization of nascent P-TEFb into the nucleus (**Fig 2A**).

203   This process is coincident with the assembly of the P-TEFb regulatory complex 7SK snRNP (**Fig**

204   **2B**). The phorbol ester ingenol, prostratin and PMA were also effective inducers of P-TEFb, but

205   not surprisingly, almost no P-TEFb was induced by the relatively ineffective HDACi SAHA or by

206   stimulation of cells with anti-CD3 alone, ionomycin or TNF-α (**Fig 1D**).

207   As shown in **Fig 1C** the expression of P-TEFb subunits and the T-loop phosphorylated

208   forms of CDK9 in both healthy donor memory CD4$^+$ T cells and resting polarized primary Th17

209   cells obtained using the QUECEL protocol (**S4A Fig**) before and after TCR activation are

210   indistinguishable. We have extensively used the highly reproducible QUECEL model to study HIV

211   latency in primary CD4+ T cells [15, 64]. In the unstimulated condition, both memory CD4$^+$ T cells

212   and resting primary Th17 cells exhibit low expression of CycT1 and the T-loop phosphorylated

213   forms of CDK9 (pSer175 and pThr186) (**Fig 1C and S2, S3, S4B Figs**). While TCR activation of

214   both cell types over an overnight period resulted in a modest elevation of CDK9 protein (by an

215   average of 1.4-fold), it also led to a substantial and proportional increase in the percentage of

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216   cells that express CycT1, pSer175 CDK9 and pThr186 CDK9 (by an average of 9-fold, 12.2-fold,

217   and 6.3-fold, respectively) (**Fig 1C**). Generation of CycT1 and the transcriptionally active marker

218   of P-TEFb, pSer175 CDK9, were observed to be quite rapid, reproducibly occurring within 2 h of

219   TCR co-stimulation and concomitant with formation of the P-TEFb-phosphorylated form of RNAP

220   II (pSer2 RNAP II CTD) (**Fig 1B and S3, S5A Figs**). These results clearly indicate that the dual

221   expression profiling of CycT1 and pSer175 CDK9 can serve as a dependable measure of

222   catalytically active and gene-associated P-TEFb in primary T cells. Therefore, in the current study

223   induction of transcriptionally active P-TEFb was assessed as the coordinate expression of CycT1

224   and pSer175 CDK9.

225   The absolute requirement for P-TEFb induction to support emergence of HIV from latency

226   in primary CD4$^+$ T cells was demonstrated by the observation that a relatively selective CDK9

227   kinase inhibitor flavopiridol (FVP) repressed proviral reactivation in latently infected primary Th17

228   cells by an average of 2.5-fold (**S5B Fig**). Therefore, HIV reactivation in latently infected primary

229   T cells is clearly preceded by and dependent on CycT1 posttranscriptional synthesis and the

230   formation of transcriptionally active P-TEFb in the nucleus in response to intracellular signals

231   emanating from TCR co-stimulation.

Regulation of 7SK snRNP and super elongation complex factors in primary T cells

233   Efficient stimulation of proviral HIV transcription elongation by P-TEFb is regulated by 7SK

234   snRNP and facilitated by the super elongation complex (SEC) (**Fig 1A**) [8, 9, 42, 43]. We therefore

235   examined the expression profiles of 7SK snRNP and SEC components in primary CD4$^+$ T cells

236   before and after activation. Western blotting of resting and TCR-activated Th17 cells

237   demonstrated notable restrictions in protein expression of not just CycT1 but also HEXIM1 and

238   the SEC components AFF1 and ELL2 during quiescence (**S6A Fig**). Transcriptome analysis (bulk

239   RNA-seq) of primary CD4$^+$ T cell types that had been polarized into the four major T-cell subsets

240   (Th1, Th2, Th17, and Treg) [64] was also conducted to provide an additional measure of the

241 differential expression of transcripts belonging to P-TEFb subunits (CDK9, CycT1 and CycT2),

242 7SK snRNP components (HEXIM1, HEXIM2, LARP7 and MEPCE) and SEC proteins (AFF1,

243 AFF2, ELL, ELL2 and ENL). By including in our analysis a publicly available bulk RNA-seq dataset

244 generated by Zhao et al. using unstimulated and TCR-activated memory CD4⁺ T cells [65] (SRA

245 accession SRP026389) (Extended data shown in **S6B-S6D Figs**), we were able to demonstrate

246 that each of these *in vitro* polarized T-cell subsets can faithfully recapitulate the phenotypic

247 characteristics of memory CD4⁺ T cells in maintaining the pattern of expression of these factors

248 (**Figs 3A-3C**). Most notably, the high-throughput sequencing studies demonstrated that there was

249 little change in CycT1 mRNA levels over a 24-h period of TCR co-stimulation (**Fig 3A and S6B**

250 **Fig**), consistent with the prevailing idea that this P-TEFb subunit is post-transcriptionally

251 regulated. Also noteworthy is the observation that HEXIM2 mRNA was very low in resting primary

252 CD4⁺ T cells (~6 transcripts per million) and was further slightly reduced following TCR co-

253 stimulation (~5 transcripts per million) (**Fig 3B**). By contrast, HEXIM1 was found to be expressed

254 at approximately 4-fold and 7-fold higher levels compared to HEXIM2 in unstimulated and 24-h

255 TCR-activated primary CD4⁺ T cells, respectively (**Fig 3B**). This finding suggests that HEXIM1 is

256 the major isoform in CD4⁺ T cells and therefore the more probable regulatory partner for P-TEFb

257 within 7SK snRNP. Consistent with our immunoblotting analysis (**S6A Fig**) and previous findings

258 that ELL2 is tightly regulated and therefore an important limiting factor for SEC formation [66, 67],

259 we observed that ELL2 mRNA is minimal in resting CD4⁺ T cells and highly inducible in response

260 to TCR activation (**Fig 3C and S6C Fig**). Single-cell RNA-seq (scRNA-seq) that were carried out

261 using both resting and TCR-activated memory CD4⁺ T cells also supported the findings from the

262 bulk RNA-seq analysis in showing a restriction of ELL2 in resting T cells and a predominance of

263 HEXIM1 over HEXIM2 (**S7A and S7B Figs**). Thus, these overall results suggest that the

264 posttranscriptional restriction in CycT1 and repression of the P-TEFb-associated factors HEXIM1,

265 AFF1 and ELL2 may need to be overcome in resting CD4⁺ T cells in order to achieve a robust

266 reactivation of HIV from latency.

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Reactivation of P-TEFb in primary T cells is not mediated by protein kinase C

Treatment with a variety of protein kinase C (PKC) agonists (ingenol, prostratin or PMA) stimulated the dual expression of CycT1 and pSer175 CDK9 in memory CD4$^+$ T cells but at lower mean levels compared to that observed with TCR co-stimulation (**Fig 1D**). Co-treatment of memory T cells with ionomycin and each of the three PKC agonists did not further elevate P-TEFb expression but substantially enhanced formation of both the transcriptionally active form of NF-κB (pSer529 p65) and the P-TEFb-modified pSer2 CTD RNAP II (**Fig 4A**). While treatment with PKC agonists elicited minimal cytotoxicity to memory T cells (relative to unstimulated cells viability was found to be 93%, 91% and 87% for ingenol, prostratin and PMA, respectively), co-treatment with ionomycin resulted in a significant loss of cell viability (35-40% cell death compared to unstimulated cells) (**Fig 4A**). These results suggest that while intracellular calcium mobilization is not essential for enabling P-TEFb biogenesis in memory T cells, it significantly potentiates the efficacy of PKC agonists at generating the transcription elongation competent form of RNAP II albeit with significant cell cytotoxicity. Exposure of polarized primary Th17 cells to ingenol or prostratin was sufficient to stimulate the formation of P-TEFb and resulted in a comparable extent of proviral reactivation in latently infected Th17 cells as TCR co-stimulation (**Fig 4B and S5D Fig**). By comparison, TNF-α and SAHA which are ineffective at generating transcriptionally active P-TEFb in both memory and Th17 cells (**Fig 1D and S4B Fig**), were found to be poor reactivators of proviral HIV in the QUECEL Th17 latency model (**S5C Fig**). Thus, the reactivation of HIV from latency appears to be tightly coupled to the biogenesis of P-TEFb in primary T cells and both of these processes can be elicited upon cellular treatment with PKC agonists in the absence of intracellular calcium mobilization.

PKC agonists, commonly referred to as phorbol esters, are structural mimics of endogenously generated diacylglycerol (DAG) and bind the C1 domains of both conventional (α, βI, βII, and γ) and novel (δ, θ, ε, and η) PKC isozymes leading to their membrane recruitment and

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292   stimulation of their kinase activities [30]. We therefore examined whether any of these DAG-

293   binding PKC enzymes mediate the expression of CycT1 and/or T-loop phosphorylated CDK9 in

294   primary T cells in response to TCR co-stimulation or phorbol ester treatment (**Fig 4C**). Healthy

295   donor memory CD4$^+$ T cells and polarized primary Th17 cells were either activated through the

296   TCR or challenged with phorbol ester in the presence or absence of treatment with a conventional

297   pan-PKC inhibitor (Ro-31-8220), an inhibitor that potently inhibits PKC α, β, γ, and δ isoforms

298   (Gö-6983), or one that selectively targets PKC-θ (sotrastaurin). PKC-θ is considered to be the

299   predominantly functional PKC isoform in T cells and it critically mediates both TCR-induced NF-

300   κB and global T-cell activation [68-70]. Surprisingly, all three PKC inhibitors failed to effectively

301   block either the TCR- or the phorbol ester-mediated inducible expression of CycT1, pSer175

302   CDK9 and pThr186 CDK9 in both memory CD4$^+$ T cells and polarized primary Th17 cells or had

303   only very modest effects in their inhibition (**Figs 5A, 5B and S8 Fig**). Similarly, these inhibitors

304   were ineffective at suppressing the reactivation of latent HIV in primary Th17 cells that was elicited

305   by either TCR co-stimulation or challenge with phorbol ester (**Figs 5B, 5C and S5E Fig**).

306   By contrast, sotrastaurin effectively inhibited the activation of NF-κB in memory T cells in

307   response to phorbol esters as measured by monitoring the formation of pSer529 p65 (**Fig 5A**). In

308   addition to serving as a positive control for the drug's activity, this observation is consistent with

309   the prevailing idea that PKC-θ is a primary mediator of NF-κB activation in T cells. We were also

310   able to demonstrate that Ro-31-8220 can disrupt the degradation of IκB-α due to TCR activation

311   in memory T cells, thereby delaying the peak mobilization of NF-κB subunits p65 and p50 into the

312   nucleus (**S9A-S9C Fig**). Overall, these results indicate that the coordinate reactivation of P-TEFb

313   and latent HIV in primary T cells in response to either TCR co-stimulation or phorbol ester

314   challenge is largely independent of PKC signaling pathways.

315   **PKC agonists signal through the MAPK ERK pathway to stimulate P-TEFb**

316   **expression and reactivate latent HIV in primary T cells**

317   TCR-mobilized diacylglycerol and exogenous phorbol esters can also target several other

318   proteins with C1 domains including RasGRP, a guanine nucleotide exchange factor for Ras in T

319   cells [29]. Membrane-anchored RasGRP1 facilitates the activity of SOS, a second Ras GDP-GTP

320   exchange factor, in a positive feedback mechanism that involves the allosteric activation of SOS

321   by RasGRP-generated Ras-GTP [71]. Exchange of GDP for GTP on Ras by both RasGRP and

322   activated SOS causes the formation of Ras–Raf complexes at the plasma membrane that lead to

323   the activation of the MAPK isoforms ERK1 and ERK2 (ERK1/2) (**Fig 6A**) [72, 73].

324   We were able to confirm that phorbol ester treatment of T cells rapidly stimulates the

325   activating phosphorylation of ERK1/2 (pThr202/pTyr204) concomitant with the inducible synthesis

326   of c-Fos and its localization to the nucleus (**S10A Fig**). Therefore, we examined whether the Ras–

327   Raf–MEK–ERK1/2 pathway (**Fig 6A**) can also mediate the biogenesis of P-TEFb in response to

328   TCR or phorbol ester challenge and if it could be a primary pathway for the reactivation of latent

329   HIV in primary CD4$^+$ T cells.

330   Treatment of Th17 and CD4$^+$ memory T cells with the MEK inhibitor U0126, which

331   effectively blocks the activating phosphorylation of ERK1/2 (**S10A and S10B Figs**), resulted in a

332   modest inhibition of TCR-induced P-TEFb expression (by an average of 1.5-fold) (**Fig 6B**). By

333   contrast, U0126 treatment much more effectively inhibited the expression of P-TEFb in both Th17

334   and memory T cells stimulated by either ingenol or prostratin (by an average of 3.7-fold and 7-

335   fold, respectively) (**Figs 6B and S11-S13 Figs**). Consistent with this observation, U0126 also

336   substantially repressed proviral reactivation in HIV-infected Th17 cells that was induced by

337   ingenol and prostratin (by an average of 2-fold and 3.7-fold, respectively) (**Fig 6C and S14 Fig**).

338   Although U0126 had a modest and variable repressive effect on TCR-induced P-TEFb

339   expression, there was a stronger repressive effect on TCR-induced proviral reactivation in HIV-

340   infected Th17 cells (1.75-fold repression of HIV compared to the 1.5-fold repression observed on

341   P-TEFb) (**Fig 6C and S14 Fig**). To test the hypothesis that TCR co-stimulation may be exerting

342 effects on the initiation of proviral transcription by generating the activator protein 1 (AP-1)

343 transcription factor complex of c-Fos and c-Jun through a crosstalk between the ERK1/2 and JNK

344 MAPK pathways (**S15A Fig**), we examined whether inhibiting JNK either individually or in

345 combination with MEK had any significant effects on proviral reactivation as well as P-TEFb

346 expression. Inhibition of JNK signaling with SP600125 caused a 1.3-fold reduction in TCR-

347 induced proviral reactivation compared to a 1.7-fold reduction observed with U0126 treatment

348 (**S15B Fig**). Treatment of latently infected Th17 cells with both inhibitors failed to elicit additional

349 suppression of TCR-induced proviral gene expression beyond that observed with U0126

350 treatment (**S16 Fig**). Similarly, inhibition of JNK did not further enhance the modest repression of

351 TCR-induced P-TEFb expression observed upon MEK inhibition (**S17 Fig**). Moreover, and not

352 surprisingly, inhibition of JNK had a very modest suppressive effect on ingenol- and prostratin-

353 induced proviral and P-TEFb expression (**S16 and S17 Figs**) clearly indicating that these phorbol

354 esters do not signal through JNK in eliciting their stimulatory effects. By contrast, a Raf inhibitor

355 AZ 628 which selectively and potently inhibits Raf1 and B-Raf kinase activities (with IC_{50} values

356 of 29 and 105 nM, respectively) was effective at suppressing P-TEFb induction in response to

357 ingenol and prostratin but had no inhibitory effect on TCR-induced P-TEFb expression (**S18 Fig**).

358 In summary, our results clearly suggest that MEKK1-MKK4/7-JNK-c-Jun is unlikely to be

359 an essential MAPK pathway for regulating the formation of P-TEFb or the reactivation of latent

360 HIV in CD4$^+$ T cells. Instead, our results strongly support the idea that the Raf-MEK-ERK1/2

361 MAPK pathway is a primary mediator of the expression of active P-TEFb in memory T cells upon

362 challenge with DAG-mimicking phorbol esters and that this signaling pathway is also essential for

363 the reactivation of latent HIV by these candidate LRAs.

364 **RasGRP1 is the predominantly expressed DAG-dependent RasGRP isoform in**

365 **primary CD4$^+$ T cells**

366   ERK1/2 activation by TCR co-stimulation is dependent on stimulation of Ras by its

367   membrane-associated guanine nucleotide exchange factors RasGRP and SOS [74]. The Ras

368   GDP-GTP exchange activity of SOS in T cells has been found to be allosterically dependent upon

369   initial activation of Ras by RasGRP1 [71]. Therefore, we hypothesized that RasGRP1 has a

370   central role in mediating the reactivation of P-TEFb and HIV in primary T cells through the ERK1/2

371   pathway upon challenge with PKC agonists.

372   The guanine nucleotide exchange activity of three of the four known isoforms of RasGRP,

373   namely, RasGRP1, RasGRP3 and RasGRP4 is known to be tightly regulated by the high affinity

374   binding of membrane-associated DAG or phorbol esters to the C1 domains of these proteins [29].

375   Upon cellular treatment with DAG-mimicking phorbol esters, RasGRP1, RasGRP3 and RasGRP4

376   rapidly translocate to cellular membranes where they contribute to the activation of membrane-

377   bound Ras [75-77]. By contrast, the C1 domain of RasGRP2 has a very poor binding affinity for

378   DAG [78, 79], and consistent with this observation, the protein fails to translocate to cellular

379   membranes in response to treatment with DAG analogs [80].

380   We therefore examined whether treatment of memory CD4+ T cells with PKC agonists can

381   trigger the recruitment of RasGRP1 to the plasma membrane. High magnification

382   immunofluorescence microscopy revealed a rapid (within 2 h) translocation of RasGRP1 to the

383   membrane following stimulation of memory T cells with ingenol, prostratin or PMA (**Fig 7A**). The

384   degree of colocalization of RasGRP1 with the plasma membrane-resident protein Ezrin in cells

385   treated with each of these three PKC agonists was comparable to that observed with TCR-

386   activated cells (**Fig 7B**).

387   In order to determine the expression profiles of each of the four RasGRP isoforms, scRNA-

388   and bulk RNA-seq were carried out in both resting and TCR-activated primary CD4+ T cells. **Fig**

389   **7C** is a UMAP profiling of memory CD4+ T cells that were either unstimulated or activated through

390   the TCR for 24 or 72 h prior to scRNA-seq. By utilizing this algorithmic analytical approach we

16

391   observed distinct clustering of cells depending on the duration of T-cell activation. Further analysis

392   of these UMAP cell clusters for specific transcript expression revealed that both RasGRP1 and

393   RasGRP2 are expressed at comparable levels in unstimulated memory CD4$^+$ T cells with barely

394   any detectable expression of RasGRP3 and RasGRP4 (**Fig 7D**). Irrespective of the duration of

395   TCR co-stimulation, RasGRP3 and RasGRP4 transcripts remained extremely low. Interestingly,

396   RasGRP2 expression was abolished upon TCR activation while RasGRP1 transcript levels were

397   maintained in these cells following 24 h TCR activation but declined slightly at 72 h post-

398   stimulation.

399   Consistent with these findings, bulk RNA-seq of primary CD4$^+$ T cell types that had been

400   polarized into the four major T-cell subsets (Th1, Th2, Th17, and Treg) showed that while

401   RasGRP3 and RasGRP4 mRNA are barely expressed in these cells regardless of the cellular

402   activation state, RasGRP2 transcript expression is highly restricted to cells that are in the

403   quiescent phase (**S19A Fig**). The sharp decline in RasGRP2 mRNA nearly abolished its

404   expression following an overnight period of TCR activation. By contrast, RasGRP1 was found to

405   be expressed at levels that were comparable to those of RasGRP2 in the resting phase of all four

406   CD4$^+$ T-cell subsets and despite the greater than 2-fold reduction in RasGRP1 upon overnight

407   TCR co-stimulation, it became the predominantly expressed RasGRP isoform in activated cells

408   (by at least 8-fold higher transcript levels) in view of the near complete abrogation of RasGRP2

409   and the extremely low expression of RasGRP3 and RasGRP4 (**S19A Fig**).

410   We performed an additional assessment of RasGRP isoform expression with a bulk RNA-

411   seq dataset generated using memory CD4$^+$ T cells that were either unstimulated or subjected to

412   a time course of TCR activation (0, 2, 4, 6, and 24 h) [65]. In agreement with our own scRNA- and

413   bulk RNA-seq experiments described above, analysis of these datasets clearly demonstrated that

414   while RasGRP3 and RasGRP4 transcripts were at very low – at times barely detectable – levels

415   across all time points examined, RasGRP2 expression was comparable to that of RasGRP1 in

17

416 the unstimulated state then exhibited a rapid and precipitous decay over the 24-h period of T-cell

417 stimulation (**S19B Fig**). By 6 h of TCR activation, RasGRP2 transcript levels had nearly dropped

418 to those of RasGRP3 and RasGRP4. By contrast, RasGRP1 transcript levels sharply declined by

419 greater than 2-fold within 2 h of TCR activation then rose slightly at the 4 h time point before

420 declining again at 24 h (**S19B Fig**). Between the 6 h and 24 h activation period RasGRP1 was

421 clearly found to be the major RasGRP isoform expressed in memory CD4$^+$ T cells.

422   Thus, our RNA-seq analysis reveals that RasGRP1 is by far the predominantly expressed

423 DAG-dependent RasGRP isoform in primary CD4$^+$ T cells. Therefore, RasGRP1 is likely to be the

424 isoform that mediates the activation of Ras-Raf-MEK-ERK1/2 signaling in response to TCR co-

425 stimulation or phorbol ester treatment. Furthermore, we have found that mRNA belonging to the

426 DAG-independent isoform RasGRP2 is only present in resting CD4$^+$ T cells with its expression

427 being rapidly and nearly abrogated upon T-cell activation.

428 **PI3K-mTORC1/2 and RasGRP1-ERK1/2 are complementary pathways that are**

429 **essential for P-TEFb biogenesis and emergence of HIV from latency**

430   Having observed that the MEK inhibitor U0126 causes a modest inhibition of TCR-induced

431 expression of P-TEFb while the Raf inhibitor AZ 628 failed to block the TCR-induced expression

432 of this transcription factor (**Fig 6B and S18 Fig**), we reasoned that there could be other TCR

433 signaling pathways that may contribute to P-TEFb biogenesis in primary T cells. Several years

434 ago, Besnard *et al.* demonstrated that the pharmacological inhibition of mTORC1 and mTORC2

435 kinase activities blocks the phosphorylation of the CDK9 subunit of P-TEFb in primary CD4$^+$ T

436 cells [81]. In the same study it was found that this blockade of CDK9 phosphorylation accounted

437 for the suppression in latent HIV reactivation by the mTOR inhibitors. However, the CDK9 target

438 sites of mTOR-dependent phosphorylation were not defined in the study.

439   Activation of both mTORC1 and mTORC2 in response to TCR co-stimulation is well

440   established to be PI3K-dependent with AKT being a central mediator of the activating crosstalk

441   between both mTORC complexes (**Fig 8A**) [82, 83]. Since mTORC1 is a critical facilitator of

442   protein synthesis [84], we reasoned that activation of the PI3K-mTORC2-AKT-mTORC1 signaling

443   pathway may contribute to generation of P-TEFb by enabling the translation of the CycT1 subunit

444   which, in line with the P-TEFb biogenesis pathway (**Fig 1A**), is a prerequisite for the subsequent

445   T-loop phosphorylation of CDK9 at both Thr186 and Ser175.

446   As shown in **Fig 8B**, TCR-induced expression of transcriptionally active P-TEFb was

447   suppressed, albeit with significant variability between experiments, upon treatment of either

448   memory CD4$^+$ T cells or polarized Th17 cells with an inhibitor of PI3K (LY294002), mTORC1

449   (Rapamycin) or a selective mTOR kinase inhibitor (Torin) that blocks both mTORC1 and

450   mTORC2 activities. Mean fold reductions in TCR-induced P-TEFb expression upon PI3K,

451   mTORC1, and mTORC1/2 inhibition were found to be 2.1, 1.9, and 2.2, respectively (**Table 1**).

452   Similarly, inhibition of AKT kinase activity with ipatasertib resulted in a mean fold reduction of 1.7

453   in TCR-induced P-TEFb expression (**Table 1 and S21A Fig**). Inconsistent with the observed

454   repressive effects on P-TEFb, inhibition of PI3K, mTORC1 and mTORC1/2 kinases elicited very

455   modest reductions in TCR-induced proviral reactivation in primary Th17 cells (**Fig 8C**). Therefore,

456   although PI3K and mTORC inhibitors were found to suppress TCR-induced P-TEFb expression

457   with substantial variability but in a statistically significant manner, they were not as effective at

458   blocking the reactivation of latent HIV.

459   Based on these results we reasoned that both the PI3K-AKT-mTORC and the Raf-MEK-

460   ERK pathways may have complementary roles in regulating TCR-induced P-TEFb biogenesis.

461   Consistent with this idea, we found that a combined inhibition of MEK and PI3K or MEK and

462   mTORC in both primary Th17 and memory CD4$^+$ T cells was much more effective at preventing

463   P-TEFb expression upon TCR co-stimulation than inhibition of the individual kinases (**Figs 9A,**

Table 1. Fold reductions in TCR-induced expression of P-TEFb and HIV following inhibition of Raf-MEK-ERK and PI3K-AKT-mTOR pathways

Kinase(s)	Inhibitor(s)	Mean +/- S.E.
MEK	10 µM U0126	1.34 +/- 0.17
	20 µM U0126	1.61 +/- 0.22
Raf	100 nM AZ 628	0.96 +/- 0.035
	500 nM AZ 628	1.16 +/- 0.039
PI3K	2.5 µM LY294002	2.14 +/- 0.40
	5 µM LY294002	3.19 +/- 0.92
AKT	1 µM ipatasertib	1.45 +/- 0.17
	10 µM ipatasertib	1.72 +/- 0.21
mTORC1	200 nM Rapamycin	1.90 +/- 0.32
	500 nM Rapamycin	1.93 +/- 0.36
mTORC1 + mTORC2	50 nM Torin	1.62 +/- 0.22
	100 nM Torin	2.20 +/- 0.30
MEK + PI3K	10 µM U0126 + 2.5 µM LY294002	5.37 +/- 0.95
	20 µM U0126 + 2.5 µM LY294002	7.83 +/- 3.82
MEK + mTORC1	10 µM U0126 + 200 nM Rapamycin	5.01 +/- 0.99
	20 µM U0126 + 200 nM Rapamycin	8.25 +/- 0.68
MEK + mTORC1/2	10 µM U0126 + 100 nM Torin	6.57 +/- 1.44
	20 µM U0126 + 100 nM Torin	8.73 +/- 1.44

Mean values are an average of fold reductions calculated as a ratio of the measure obtained for TCR activation over that obtained for TCR activation in the presence of inhibitor(s). S.E.: standard error of the mean.

20

483   **9B and S22 Fig, Table 1**). Therefore, the combined treatment of these cells with U0126 and

484   LY294002 or U0126 and either mTORC inhibitor (rapamycin for mTORC1 or Torin for mTORC1

485   and mTORC2) nearly abrogated TCR-induced P-TEFb expression and with minimal cytotoxicity

486   (**Figs 9A and 9B**).

487   We also examined the effect of these combined treatments on proviral expression

488   following TCR co-stimulation of latently infected primary Th17 cells. Inhibition of both mTORC1

489   and mTORC2 with Torin was most effective in combination with U0126 at suppressing proviral

490   reactivation beyond that observed with either inhibitor (**Fig 9C and S23 Fig**). Overall, these results

491   indicate that two of the major signaling arms of the TCR cascade, namely, RasGRP1–Ras–Raf–

492   MEK–ERK1/2 and PI3K–mTORC2-AKT–mTORC1 serve essential complementary functions in

493   mediating the biogenesis of P-TEFb in primary CD4^{+} T cells that is in turn crucial to enabling the

494   emergence of HIV from latency.

Discussion

Regulation of P-TEFb in primary T cells is central to HIV transcriptional control

497   Dissecting the cellular pathways through which HIV emerges from latency is critical to the

498   development of "shock and kill" therapeutic approaches that can be used to effectively eradicate

499   persistent reservoirs of transcriptionally latent but replication-competent HIV proviruses in

500   memory CD4^{+} T cells of infected individuals. Using the well-characterized and highly reproducible

501   primary QUECEL model of HIV latency together with healthy donor memory CD4^{+} T cells, we

502   identified a series of T-cell signaling pathways that are crucial to the emergence of HIV from

503   latency. In particular, the host transcription elongation factor P-TEFb is an obligate activator of

504   proviral HIV transcription, and therefore, comprehensively understanding its regulation by signal

505   transduction mechanisms in primary T cells is central to HIV cure efforts.

506   P-TEFb is expressed at vanishingly low levels in memory CD4$^+$ T cells and this is one of

507   the key regulatory mechanisms that minimizes HIV transcription in resting cells. However, TCR

508   co-stimulation initiates a set of signaling cascades that result in the posttranscriptional expression

509   of its CycT1 subunit and T-loop phosphorylation of CDK9 kinase at Ser175 and Thr186.

510   Therefore, the studies presented here were specifically aimed at identifying precise TCR signaling

511   pathways in primary T cells that are essential for the generation of transcriptionally active P-TEFb,

512   defined as the coordinate expression of CycT1 and pSer175 CDK9.

513   Our major findings, as summarized in the scheme shown in **Fig 10**, are: i) reactivation of

514   HIV from latency is tightly coupled to the biogenesis of P-TEFb and both of these processes can

515   be elicited upon cellular treatment with PKC agonists in the absence of intracellular calcium

516   mobilization with an ionophore stimulus; ii) stimulation of both P-TEFb expression and proviral

517   reactivation upon TCR co-stimulation or challenge with PKC agonists is largely independent of

518   PKC activity; iii) PKC agonists signal through off-target activation of the RasGRP1-Ras-Raf-MEK-

519   ERK1/2 to stimulate both P-TEFb biogenesis and the reactivation of latent HIV; and iv) generation

520   of transcriptionally active P-TEFb upon TCR co-stimulation is primarily mediated by two

521   complementary pathways, namely, the RasGRP1-Ras-Raf-MEK-ERK1/2 and the PI3K–

522   mTORC2-AKT–mTORC1 pathways.

523   **Off-target pathway mediating P-TEFb induction by PKC agonists**

524   T-cell challenge with a PKC agonist (usually PMA) plus a calcium ionophore has been

525   widely used in the HIV cure field as a gold standard for measuring the relative effectiveness of

526   LRAs at reversing HIV latency in both *in vitro* primary cell models and patient-derived CD4$^+$ T

527   cells [85-87]. In our current study using the QUECEL primary T-cell HIV latency model, we found

528   that single challenge with ingenol or prostratin reproducibly elicited levels of proviral reactivation

529   that were comparable to TCR co-stimulation. Each of the PKC agonists we examined was able

530   to stimulate active P-TEFb expression in both primary Th17 and memory T cells, albeit not as

531   effectively as TCR co-stimulation. However, co-treatment with ionomycin failed to improve their

532   stimulatory effects on P-TEFb. Moreover, ionomycin treatment on its own at best modestly

533   induced CycT1 and pSer175 CDK9 expression. These findings strongly suggest that the calcium

534   signaling arm of the TCR pathway may not crucial for the inducible expression of P-TEFb in these

535   cells.

536   Calcium signaling has been shown to be essential for the initiation of HIV transcription in

537   the QUECEL model via activation of NFAT [64]. However, it seems likely that under conditions of

538   phorbol ester treatment alone HIV transcription initiation is mediated primarily by NF-κB as is seen

539   in the Jurkat T-cell models [35, 88, 89]. Co-treatment of memory and Th17 cells with ionomycin

540   significantly potentiated the efficacy of PKC agonists at generating the transcriptionally active

541   form of NF-κB (pSer529 p65) and the transcription elongation competent form of RNAP II (pSer2

542   CTD RNAP II), which provides a downstream readout of P-TEFb enzymatic activity, since pSer2

543   CTD RNAP II is a modification that is at least in part due to P-TEFb and is strictly associated with

544   polymerase that is already present on transcribing genes [90, 91]. It is therefore quite likely that

545   calcium signaling serves to promote the recruitment of RNAP II to gene promoters in manner that

546   is dependent upon the activation of both NF-κB and NFAT. It is also noteworthy that single

547   challenges with the PKC agonists were minimally cytotoxic to primary T cells while the co-

548   treatment with ionomycin resulted in a significant loss of cell viability. These observations are

549   consistent with a recent report showing that treatment of CD4$^+$ T cells with prostratin or byrostatin-

550   1 induces resistance to apoptosis in a PKC-dependent manner [92]. Thus, because of their ability

551   to induce P-TEFb biogenesis and stimulate the reactivation of HIV from latency, more selective

552   PKC agonists show strong potential as candidate LRAs.

553   It is generally believed that the latency reversal activity of PKC agonists, also commonly

554   referred to as phorbol esters is due to the activation of PKC-dependent pathways. These agents

555   structurally mimic endogenously generated DAG to bind the C1 domains of both conventional (α,

23

556   βI, βII, and γ) and novel (δ, θ, ε, and η) PKC isozymes leading to their membrane recruitment and

557   stimulation of their kinase activities [30]. However, they can also target several other proteins with

558   C1 domains including RasGRP, a critical guanine nucleotide exchange factor for Ras in T cells,

559   α and β chimaerins, Munc13 proteins, protein kinase D (PKD) and DAG kinases (DGKs) [93]. In

560   our current study, we unexpectedly found that pan-PKC inhibitors (which potently inhibit α, β, γ,

561   δ, and ε isoforms) and an inhibitor that selectively targets PKC-θ, considered to be the

562   predominantly functional PKC isoform in T cells [68-70], were all ineffective at blocking phorbol

563   ester- or TCR-induced P-TEFb expression and proviral reactivation in primary T cells. By contrast,

564   selective inhibitors of Raf and MEK kinases effectively suppressed the induction of P-TEFb that

565   was in response to phorbol ester treatment. Moreover, the MEK inhibitor also effectively

566   suppressed phorbol ester-induced proviral reactivation in the QUECEL model. These results

567   strongly indicate that these agents are signaling through the Ras-Raf-MEK-ERK pathway rather

568   than through PKC in eliciting their stimulatory activity on P-TEFb and HIV in primary T cells.

RasGRP1 is a key mediator of P-TEFb biogenesis

570   A critical initiator of Ras-Raf-MEK-ERK MAPK signaling in response to cellular treatment

571   with DAG-mimicking phorbol esters is the Ras GDP-GTP exchange factor RasGRP [29, 74]. Of

572   the four known isoforms of RasGRP, RasGRP1, RasGRP3 and RasGRP4 have been shown to

573   bind phorbol esters with high affinity via their C1 domains and, consequently, they get recruited

574   to the plasma membrane where they are able to access Ras and facilitate guanine nucleotide

575   exchange [29, 75-77]. SOS, another important Ras GDP-GTP exchange factor, does not bind

576   DAG but is rather anchored to the membrane by a complex of tyrosine-phosphorylated LAT and

577   the adaptor protein Grb2 [94]. Following T-cell stimulation guanine nucleotide exchange activity

578   of membrane-associated SOS is triggered by the initial activation of Ras by RasGRP [71]. In turn

579   GTP-bound Ras allosterically binds SOS leading to its full activation. This creates a positive Ras-

24

580   GTP-SOS feedback loop that results in a conformational change in Ras which permits robust

581   formation of Ras-Raf complexes at the membrane [71, 95].

582   Transcriptome analyses of QUECEL T-cell subsets and memory CD4+ T cells performed

583   in our study using scRNA- and bulk RNA-seq approaches, unequivocally demonstrated that of

584   the three DAG-dependent RasGRP isoforms, RasGRP1 is by far the predominantly expressed

585   isoform in resting and TCR-activated cells. By contrast, RasGRP3 and RasGRP4 transcripts were

586   barely detected under both conditions. Assuming mRNA copy numbers of these isoforms

587   appropriately reflect their relative protein expression and potential activities, it is quite likely that

588   the activation of ERK MAPK signaling following TCR co-stimulation or challenge with PKC

589   agonists is being mediated by RasGRP1. Furthermore, we were able to confirm by

590   immunofluorescence microscopy that RasGRP1 rapidly translocates to the membrane following

591   stimulation of memory T cells with PKC agonists. Therefore, these overall results implicate

592   RasGRP1 as being the Ras GDP-GTP exchange factor that orchestrates the regulation of P-

593   TEFb biogenesis in primary T cells.

594   Unlike the DAG-dependent RasGRP proteins, RasGRP2 has a very poor binding affinity

595   for DAG and fails to translocate to the plasma membrane in response to treatment with DAG

596   analogs [78-80]. Membrane trafficking of RasGRP2 has been shown to be dependent on F actin

597   polymerization [96]. Moreover, a longer splice variant of RasGRP2 is found to be myristoylated

598   and palmitoylated at its N-terminus which also enables a DAG-independent membrane

599   localization [97]. Our transcriptome studies showed that RasGRP2 transcripts are highly restricted

600   to resting primary T cells. Although RasGRP2 mRNA expression was initially comparable to that

601   of RasGRP1 in the unstimulated state, it rapidly and precipitously decayed following TCR

602   activation eventually reaching the extremely low levels seen with RasGRP3 and RasGRP4.

603   Dramatic changes in the abundance of a transcript tend to correlate strongly with changes in the

604   abundance of its protein as we have observed in the case of ELL2 and HEXIM1 (**S6 Fig**).

25

605 Therefore, these RNA-seq results could be an indication that RasGRP2 is functionally relevant in

606 the context of resting T cells or during the earlier period of T-cell activation.

607 Our findings are noteworthy in view of the established role of RasGRP2 as the primary

608 activator of the Ras homolog Rap1 [97, 98], which has been demonstrated in multiple studies to

609 be capable of antagonizing Raf-activating Ras activity in T cells [99-101]. Interestingly, TCR

610 stimulation of primary human T cells with anti-CD3 in the absence of co-stimulatory signals was

611 shown to rapidly and transiently activate Rap1 (within 10 min of receptor crosslinking with anti-

612 CD3) while co-stimulation with anti-CD28 abolished the anti-CD3-dependent activation of Rap1

613 [102]. As shown in **S19C and S19E Figs**, both unstimulated memory T cells and QUECEL T-cell

614 subsets expressed much higher copy numbers of Rap1A and Rap1B transcripts as compared to

615 those belonging to H-Ras, K-Ras and N-Ras. These observations warrant further research

616 because they hint at a possible RasGRP2-Rap1 functional interaction that may modulate

617 formation of P-TEFb perhaps by counteracting RasGRP1-Ras-Raf signaling in primary T cells.

618 **Combined roles of the RasGRP1-Ras-Raf-MEK-ERK1/2 and PI3K-mTORC2-AKT-**

619 **mTORC1 pathways in mediating P-TEFb biogenesis**

620 Molecular mechanisms through which the RasGRP1-Ras-Raf-MEK-ERK1/2 pathway

621 effects the formation of transcriptionally active P-TEFb in primary T cells are still yet to be defined.

622 Using a Jurkat T-cell model system for HIV latency, we had previously shown that ERK MAPK

623 signaling in response to phorbol ester or TCR co-stimulation can serve as a primary mediator of

624 the dissociation of P-TEFb from 7SK snRNP [36]. By conducting chromatin immunoprecipitation

625 studies we were able to further demonstrate that inhibition of ERK activation by MEK substantially

626 suppressed HIV transcription elongation by preventing the recruitment of P-TEFb to latent

627 proviruses. MEK inhibition also suppressed the T-loop phosphorylation of CDK9 at Ser175 in

628 Jurkat T cells, a modification that is exclusively found on P-TEFb that is not associated with 7SK

26

629   snRNP [60]. In both of these studies we were unable to investigate whether this pathway is also

630   essential for the biogenesis of P-TEFb since Jurkat T cells constitutively express steady-state

631   levels of the enzyme, the majority of which is already assembled into 7SK snRNP.

632   Since the posttranscriptional synthesis of CycT1 is essential for initiating the assembly of

633   enzymatically active P-TEFb, as proposed in **Fig 1**, determining the exact mechanisms by which

634   ERK MAPK signaling orchestrates the translation of CycT1 in primary T cells will be of paramount

635   importance in our future studies. Two possible mechanisms may involve relieving the microRNA-

636   directed restriction of CycT1 protein synthesis in resting T cells or facilitating the nuclear export

637   of the RNA binding protein NF90. The latter was previously shown to be capable of binding the 3'

638   untranslated region of CycT1 mRNA in latently infected monocyte-derived U1 cells in order to

639   promote translation initiation of CycT1 and subsequent induction of proviral HIV [103].

640   Our identification of PI3K–mTORC2-AKT–mTORC1 as a complementary TCR pathway

641   for stimulating P-TEFb expression suggests that mTORC1 signaling can participate in P-TEFb

642   biogenesis by enhancing the translation of CycT1 mRNA. In mammalian cells, stimulation of

643   mTORC1 activity is critical to the rapid initiation of protein synthesis through the sequential

644   phosphorylation and activation of components of the translational machinery which include the

645   ribosomal protein S6 kinase 1 (S6K1), its downstream substrate ribosomal protein S6 as well as

646   initiation and elongation factors [104]. Importantly, there is a precedent for a crosstalk between

647   ERK and mTORC signaling leading up to the activation of these translation factors. A study

648   investigating the basis for cardiac hypertrophy reported that Gq protein-coupled receptor agonists

649   induce protein synthesis in cardiomyocytes by stimulating the phosphorylation of S6K1 and

650   eIF4E-binding protein-1 (4E-BP1) via Ras-ERK signaling rather than through AKT and this ability

651   is effectively blocked either by inhibition of MEK or mTORC1 [105]. It will therefore be interesting

652   to examine whether stimulation of ERK activity in primary T cells with PKC agonists may in turn

653   lead to stimulation of mTORC1 and phosphorylation of translation factors.

654   Disulfiram (DSF), an inhibitor of acetaldehyde dehydrogenase that is used for the

655   treatment of alcoholism, was shown to reactivate latent HIV-1 expression in a primary cell model

656   of virus latency and functions by depleting PTEN protein expression [106]. PTEN lipid

657   phosphatase and GSK3β kinase are well established negative regulators of the PI3K–mTORC2-

658   AKT–mTORC1 signaling pathway (**Fig 8A**) [107, 108]. However, in our study we found that

659   inhibition of their activities either individually or in tandem was not sufficient to elicit P-TEFb

660   expression in memory T cells (**S21 Fig**). Therefore, in future studies we will be testing to see

661   whether various combination treatments that involve using inhibitors of PTEN, GSK3β,

662   diacylglycerol kinase-α (DGK-α; which as shown in **S20A** and **S20B Figs** is the predominant DGK

663   isoform in primary CD4$^+$ T cells) as well as membrane-permeable diacylglycerols can stimulate

664   P-TEFb biogenesis.

Conclusions

666   Despite extensive small molecule screens, it has been challenging to identify potent latency

667   reversing agents. It seems likely that this is because there are multiple barriers to HIV reactivation

668   in latently infected cells involving both transcription initiation, transcription elongation and

669   epigenetic regulation. Despite this complexity, the major rate-limiting step for HIV transcription

670   appears to involve P-TEFb activation. Our studies have shown that although P-TEFb biogenesis

671   after TCR stimulation is the result of multiple complementary signaling pathways, effective

672   activation can be achieved through the RasGRP1-Raf-ERK1/2 pathway. It therefore seems likely

673   that selectively activating this pathway in combination with activation of a transcription initiation

674   factor, for example using a non-canonical activator of NF-κB [109], provides an optimal strategy

675   for latency reversal in the absence of full T-cell activation – the key missing element in the "shock

676   and kill" HIV eradication strategy.

Materials and Methods

678   **Materials**

679   RPMI 1640 medium and fetal bovine serum for culturing memory CD4$^+$ T cells and

680   preparing polarized Th17 cells were purchased from Hyclone. Human T-cell activator anti-CD3

681   and anti-CD28 antibody cocktail covalently bound to magnetic beads (Dynabeads) was obtained

682   from ThermoFisher Scientific. Soluble anti-CD3 and anti-CD28 antibodies were from BD

683   Biosciences. Ingenol-3-angelate and SAHA were purchased from Cayman Chemical.

684   Recombinant TNF-α was from R&D Systems. Prostratin, PMA and ionomycin were from Millipore

685   Sigma. The sources and catalog information of all commercially obtained inhibitors and antibodies

686   used in the current study are shown in **S1 Table**. HEXIM1 antibody was custom-synthesized and

687   affinity purified by Covance Research Products. Generation, purification and validation of the

688   polyclonal antibody towards the phospho-Ser-175 CDK9 epitope has been described previously

689   [60].

690   **Isolation and experimental culture of memory CD4$^+$ T cells**

691   Approximately 1.0 X 10^7 memory CD4$^+$ T cells were routinely isolated by negative bead

692   selection from previously frozen healthy donor leukapheresis packs containing 1.0 X 10^8

693   peripheral blood mononuclear cells (PBMCs) using the EasySep^{TM} memory CD4$^+$ T-cell

694   enrichment kit (StemCell). After isolation cells were allowed to recover overnight in complete

695   RPMI media lacking IL-2 prior to treating them with inhibitors and/or stimuli described in the

696   current study. Purity and resting status of the isolated memory T cells were confirmed by

697   immunofluorescence flow cytometry analysis of CD45-RO, cyclin D3 and Ki67 expression. For

698   the inhibitor experiments, memory T cells were equally divided into a 24-well plate at

699   approximately 3.0 X 10^5/ml of complete media and pretreated with the appropriate inhibitors for

700   30 min before challenging them with either anti-CD3/anti-CD28 Dynabeads at a 1:1 bead-to-cell

701   ratio or with the phorbol esters ingenol-3-angelate, prostratin or PMA. For cell viability

702   assessment, experimentally treated cells were harvested by centrifugation at 1500 rpm for 3 min

29

703   in polystyrene 5-ml round-bottomed flow cytometry tubes, washed with 1X PBS and then

704   subjected to staining with eFluor450 Viability Dye (ThermoFisher Scientific) for 30 min on ice.

Preparation of quiescent and HIV-infected Th17 cells

706   The procedure for generating quiescent and/or latently infected primary Th17 cells has

707   been extensively described previously [64]. Briefly, naïve CD4$^+$ T cells isolated from previously

708   frozen healthy donor leukapheresis packs by negative magnetic bead selection using the

709   EasySep^{TM} Naïve CD4$^+$ T-cell isolation kit (StemCell) were treated for 8 days with 10 μg/ml

710   Concavanalin A and a cocktail of polarizing cytokines and antibodies. On Day 4, IL-2 was added

711   to the cells at 60 IU/ml. Following Th17 polarization and T-cell expansion, cells were infected with

712   a VSVG-pseudotyped pHR'-Nef+-CD8a/GFP viral construct by spinoculation. On Day 14, HIV

713   infected cells were isolated by anti-CD8a magnetic separation. To generate quiescent T cells,

714   both the infected and the uninfected (negatively sorted) cells were cultured in medium containing

715   reduced concentrations of IL-2 (15 IU/ml) and IL-23 (12.5 μg/ml) for at least 2 weeks. Entry into

716   quiescence was monitored by flow cytometry by measuring EdU incorporation and analyzing the

717   expression of cyclin D3 and Ki67. Proviral latency was also monitored by assessing the

718   expression of Nef by immunofluorescence flow cytometry before and after TCR reactivation for

719   24 h.

Immunofluorescence flow cytometry

721   Experimentally treated primary Th17 or memory CD4$^+$ T cells were harvested by

722   centrifugation at 1500 rpm for 3 min in polystyrene 5-ml round-bottomed flow cytometry tubes.

723   Thereafter, cells were washed with 1X PBS prior to fixation in 4% formaldehyde for 15 min at

724   room temperature. For surface-staining with fluorophore-conjugated CD45-RO, CCR7, CD25 and

725   CD69 antibodies, fixed cells were first washed with 1X PBS then incubated with these antibodies

726   for 45 min in the dark and at room temperature. For intracellular staining, fixed cells were

727   permeabilized by a sequential treatment with 0.2% Triton-X-100 for 10 min and incubation with

30

728   1X BD Perm/Wash buffer (BD Biosciences) for 15 min at room temperature. Following a 15-min

729   blocking step with a non-specific IgG, permeabilized cells were immunostained for 45 min in the

730   dark and at room temperature with fluorophore-conjugated antibodies against cyclin D3, Ki67,

731   total CDK9, cyclin T1, pThr186 CDK9, pSer175 CDK9, pSer2 CTD RNA polymerase II, or

732   pSer529 p65 NF-κB. For antibodies that were unconjugated, Alexa Fluor™ antibody labeling kits

733   from ThermoFisher Scientific were used for fluorophore conjugation. After immunostaining cells

734   were rinsed with 1X PBS and then subjected to flow cytometry analysis using the BD LSR

735   Fortessa instrument (BD Biosciences) equipped with the appropriate laser and filters.

Immunofluorescence deconvolution microscopy

737   Approximately 3.0×10^5 experimentally treated memory CD4$^+$ T cells were harvested by

738   centrifugation at 1500 rpm for 3 min, washed with 1X PBS and resuspended in 30 μl 1X PBS.

739   Resuspended cells were then placed onto poly-L-lysine-coated coverslips in a 24-well plate and

740   allowed to adhere to the surface at 37°C for 15 min. Thereafter, cells were fixed with 4%

741   formaldehyde for 15 min, washed with 1X PBS and then permeabilized with a sequential

742   treatment of 0.2% Triton-X-100 for 10 min followed by 1X Perm/Wash buffer for 30 min at room

743   temerature. Blocking was performed using 10% Normal Donkey Serum (The Jackson Laboratory)

744   prepared in 1X BD Perm/Wash for 30 min and, afterwards, cells were immunostained for 2 h at

745   room temperature with primary antibodies against CDK9, Cdc37, cyclin T1, RasGRP1 or Ezrin.

746   Primary antibody solutions were all prepared in blocking buffer. Thereafter, cells were washed

747   three times with 1X Perm/Wash buffer and then incubated for 1 h with 1:200 dilutions of

748   fluorophore conjugated anti-rabbit or anti-mouse IgG secondary antibodies in blocking buffer.

749   After washing the coverslips with 1X Perm/Wash buffer, they were counterstained with 1 μg/ml

750   DAPI for 15 min, mounted onto glass slides with ProLong Antifade medium (ThermoFisher

751   Scientific), and imaged at high magnification (100X) using a DeltaVision epifluorescent

752   microscope (Applied Precision). Images were captured in a z stack, deconvolved and processed

753   using the softWoRx analysis program (Applied Precision). For RasGRP1 and Ezrin

754   immunostaining, the softWoRx colocalization module was used to create scatterplots and

755   measure the Pearson's correlation coefficient. A region of interest for the colocalization analysis

756   was defined as a single cell. Processed images were exported as JPEG files and micrographs

757   were composed using Adobe Photoshop.

758   **Combined HEXIM1 immunofluorescence and 7SK RNA FISH staining**

759   Memory CD4$^+$ T cells that were washed and resuspended in 1X PBS as described above

760   were placed onto poly-L-lysine-coated coverslips in a 24-well plate and allowed to adhere to the

761   surface at 37°C for 15 min. Thereafter, cells were fixed with 4% formaldehyde for 15 min, washed

762   with 1X PBS and then permeabilized with a sequential treatment of 0.2% Triton-X-100 for 10 min

763   followed by 1X Perm/Wash buffer for 30 min. For the blocking step, cells were incubated in

764   blocking solution for 30 min that was prepared as follows: 1% BSA (10 μg/ml) and 50 μg/ml

765   Donkey IgG in 1X Perm/Wash. This blocking buffer was also used to prepare the primary and

766   secondary antibody solutions. After immunostaining the cover slips were washed twice with 1X

767   PBS and then fixed in 4% formaldehyde solution for 15 min at room temperature. Coverslips were

768   washed once with 1X PBS prior to incubation with Wash Buffer A (2X SSC and 10% formamide

769   in RNase-free ultrapure H$_2$O) for 5 min at room temperature. For RNA FISH, fluorophore-

770   conjugated oligonucleotide probe was resuspended in hybridization buffer (10% dextran sulfate,

771   2X SSC and 10% formamide in RNase-free ultrapure H$_2$O) to a final concentration of 125 nM.

772   Coverslips were inverted onto 40 μl droplets of the hybridization mixture in a humidified chamber

773   which was then tightly sealed and incubated in the dark at 37°C for 16 hours. Thereafter,

774   coverslips were transferred onto a fresh 24-well plate cells side up, incubated in the dark with

775   wash buffer A for 30 min and then counterstained for 15 min with 1 μg/ml DAPI that was prepared

776   in Wash Buffer A. Afterwards, the coverslips were washed twice with Wash Buffer B (2X SSC in

777   RNAase-free ultrapure H$_2$O) prior to mounting onto glass slides using Prolong Antifade medium.

778   Images were captured at high magnification (100X) by deconvolution microscopy and then

779   processed as described above.

Subcellular fractionation of primary Th17 cells for Western blotting

781   Low-salt and chromatin nuclear fractions were prepared as described previously [25].

782   Briefly, unstimulated and TCR-activated primary Th17 cells were harvested and washed with ice-

783   cold 1X PBS. Cells were then resuspended in low-salt Cytosolic Extract (CE) lysis buffer (10 mM

784   KCl, 10 mM MgCl2, 0.5% NP-40, 1 mM DTT, and 10 mM Hepes, pH 7.8) containing an EDTA-

785   free protease and phosphatase inhibitor mixture (Roche Applied Science) and placed on ice for

786   30 min. Thereafter, nuclei were gently pelleted by centrifugation at 4000 rpm for 5 min and washed

787   once with CE buffer. The nuclear pellets were placed in Cell Lysis Buffer A (150 mM NaCl, 10

788   mM KCl, 1.5 mM MgCl$_2$, 0.5% NP-40, 1 mM DTT, 10 mM Hepes pH 8.0) containing a cocktail of

789   protease and phosphatase inhibitors and lysed in multiple quick freeze/thaw cycles using

790   isopropyl alcohol/dry ice and a 37°C water bath. This was followed by sonication using a microtip

791   to mechanically shear DNA and solubilize chromatin-bound proteins. Nuclear extracts were then

792   centrifuged at 4000 rpm for 3 min to remove insoluble debris. Protein concentration in both low-

793   salt and chromatin nuclear fractions was determined at 280 nm using a Nanodrop

794   spectrophotometer (ThermoFisher Scientific). Thereafter, both fractions were subjected to

795   Western blotting using primary antibodies towards AFF1, ELL2, ENL, CDK9, cyclin T1, HEXIM1,

796   p65, p50, NFAT-c1, c-Fos and c-Jun.

Single-cell transcriptome profiling of memory CD4^{+} T cells

798   Memory CD4^{+} T cells were isolated from a healthy donor leukapheresis pack and allowed to

799   recover overnight in complete RPMI medium with no IL-2 addition prior to challenging them or not

800   with anti-CD3/anti-CD28 Dynabeads for 24 or 72 h at a 1:1 bead-to-cell ratio in media containing

801   15 IU/ml IL-2. Thereafter, cells were prepared for Drop-seq by magnetically removing the T-cell

802   receptor activator beads, removal of dead cells using the MACS dead cell removal kit and

33

803   resuspension of cells in 1X PBS containing 0.1% BSA at 2 X 10^5 cells/ml. To perform

804   transcriptome profiling, a total of 600,000 single cells from each of the three samples were

805   subjected to Drop-seq. Briefly, cells were resuspended in PBS/1% BSA at 200,000 cells/ml. Drop-

806   seq was performed as previously described [110] using microfluid device (FlowJEM, Toronto,

807   Canada) and barcoded beads (ChemGenes, Inc.). After the isolation of single cells with barcoded

808   beads in oil droplets (Bio-Rad, Inc.), we prepared indexed cDNA libraries using Nextera XT library

809   preparation kit (Illumina, Inc.). Libraries were sequenced using Illumina HiSeqX (Medgenome,

810   Inc.). We mapped the resulting reads against the human GRCh38.p13 reference genome and

811   used the Drop-Seq Toolkit v. 1.0 to generate the digital gene expression (DGE) matrices. Our

812   primary data is publicly available at GEO (Accession no. GSE167916). We analyzed, visualized

813   and explored the resulting DGE matrices using an open-source Seurat software package for R,

814   version 4 [111-113]. Each DGE matrix was used to generate a Seurat object. Each single-cell

815   transcription profile taken for further analysis expressed at least 100 genes. We used the

816   SCTransform protocol to normalize the data. Common anchor features (genes) were identified

817   and used to combine the data from different samples into a single Seurat object. After the

818   identification of highly variable genes, principal component analysis (PCA) was performed.

819   Significant principal components were determined using the Jackstraw plot analysis. To classify

820   distinct groups of cells we performed the dimensionality reduction using the Uniform Manifold

821   Approximation and Projection (UMAP) algorithm [114]. Cells were clustered according to their

822   unbiased transcriptome signatures (unsupervised clustering). We used the Dot-plot function in

823   Seurat to quantify positive cell enrichment and relative expression levels of genes of interest in

824   the dataset.

825   **Bulk RNA-seq of primary CD4+ T-cell QUECEL subsets**

826   Bulk RNA-seq analysis was performed on cells in fully quiescent state and 24 hours

827   following reactivation according to the QUECEL protocol [64] (SRA accession SRP145508). RNA-

828   seq reads were quality controlled using Fastqc and trimmed for any leftover adaptor-derived

829   sequences, and sequences with Phred score less than 30 with Trim Galore, which is a wrapper

830   based on Cutadapt and FastQC. Any reads shorter than 40 nucleotides after the trimming was

831   not used in alignment. The pre-processed reads were aligned to the human genome

832   (hg38/GRCh38) with the Gencode release 27 as the reference annotations using STAR version

833   2.7.2b [115], followed by gene-level quantitation using htseq-count [116]. In parallel, the pre-

834   processed reads were pseudoaligned using Kallisto version 0.43.1 [117] with 100 rounds of

835   bootstrapping to the Gencode release 27 of the human transcriptome to which the sequence of

836   the transfected HIV genome and the deduced HIV spliced transcripts were added. The resulting

837   quantitations were normalized using Sleuth. The two pipelines yielded concordant results.

838   Transcripts per million (TPM) values were used to evaluate the relative abundance of transcripts

839   under resting and activated conditions. A publicly available bulk RNA-seq dataset of primary

840   human memory CD4$^+$ T cells that had been activated or not through TCR co-stimulation (SRA

841   accession SRP026389) was subjected to an identical analysis workflow as described above.

Acknowledgements

843   We thank all past and present members of the Karn laboratory for their help and useful

844   discussions. We also thank the CWRU/UH Center for AIDS Research for the provision of

845   microscopy and flow cytometry services.

Figure legends

847   **Fig 1. Stimulation of P-TEFb assembly in memory CD4$^+$ T cells by TCR co-stimulation and**

848   **PKC agonists.** *(A)* Proposed scheme for the biogenesis of P-TEFb in memory CD4$^+$ T cells and

849   its exchange from 7SK snRNP to assemble the super elongation complex (SEC) on proviral HIV.

850   Posttranscriptional synthesis of cyclin T1 (CycT1) initiated immediately upon TCR co-stimulation

851   serves as a trigger for the heterodimeric assembly of P-TEFb and T-loop phosphorylation of CDK9

852   kinase at Thr186. Assembled P-TEFb is translocated into the nucleus where it is associated with

853   HEXIM1 and 7SK snRNP to form 7SK snRNP. Exchange of P-TEFb from 7SK snRNP is facilitated

854   by the displacement of HEXIM1 by Tat and also T-loop phosphorylation of CDK9 at Ser175

855   leading to formation of the super elongation complex (SEC) containing transcriptionally active P-

856   TEFb. This complex is then loaded onto TAR RNA to stimulate proviral transcription elongation.

857   Signaling pathways responsible for the biogenesis of P-TEFb leading up to formation of

858   transcriptionally active P-TEFb will be examined in this study. *(B)* Assessment of active P-TEFb

859   expression by immunofluorescence flow cytometry as measured by monitoring the co-expression

860   of cyclinT1 and pSer175 CDK9. *(C)* Flow cytometry analysis of the intracellular fluorescence

861   immunostaining of total CDK9, cyclin T1 (CycT1), pThr186 CDK9 and pSer175 CDK9 from at

862   least five different experiments performed using either primary Th17 (red) or memory T cells

863   (black) that were activated or not for 18 h through the TCR with anti-CD3/anti-CD28 Dynabeads.

864   *(D)* Assessment of stimuli capable of inducing P-TEFb expression in memory CD4$^+$ T cells. The

865   graph shows immunofluorescence flow cytometry measurements of the coordinate induction of

866   CycT1 and pSer175 CDK9 expression in memory T cells derived from at least four different

867   healthy donors. Cells were subjected to 24 h treatment with the indicated stimuli prior to

868   immunofluorescence staining for CycT1 and pSer175 CDK9.

869

870   **Fig 2. Imaging the nuclear assembly of P-TEFb and 7SK snRNP in activated memory CD4$^+$**

871   **T cells.** *(A)* Subcellular expression of P-TEFb subunits in memory CD4$^+$ T cells before and after

872   T-cell receptor (TCR) activation. Cells were stimulated or not with a soluble cocktail of anti-

873   CD3/anti-CD28 antibodies for 4 or 24 h prior to dual immunostaining for CDK9 and CycT1. Images

874   were captured at 100X using a deconvolution microscope. Scale bar: 10 μm. *(B)* Visualization of

875   the expression and subcellular localization of 7SK snRNA and HEXIM1 in memory CD4$^+$ T cells.

876   These imaging experiments were carried out by combining RNA FISH with immunofluorescence

877   staining of unstimulated memory T cells, cells activated through the TCR for 2 or 24 h with anti-

878   CD3/anti-CD28 Dynabeads, or cells challenged with a combination of the PKC agonist prostratin

36

879   and the calcium ionophore ionomycin. Images were captured at 100X by deconvolution

880   microscopy. Scale bar: 5 µm.

881

882   **Fig 3. Transcriptome analysis of P-TEFb, 7SK snRNP and SEC expression in primary**

883   **QUECEL subsets and memory CD4⁺ T cells.** Quiescent CD4⁺ T cells that had been polarized

884   into Th1, Th2, Treg and Th17 subsets using the QUECEL procedure were activated or not for 24

885   h with anti-CD3/anti-CD28 Dynabeads. Bulk RNA-seq datasets obtained using these cells were

886   analyzed to examine the expression of P-TEFb subunits *(A)*, 7SK snRNP components *(B)*, and

887   factors belonging to the super elongation complex *(C)*. Transcripts per million (TPM) values were

888   used to evaluate the relative abundance of transcripts under resting and activated conditions. In

889   *A*, the expression of CD25 was examined as a positive control. A publicly available bulk RNA-seq

890   dataset of primary human memory CD4⁺ T cells that had been activated or not through TCR co-

891   stimulation (SRA accession SRP026389) with anti-CD3/anti-CD28 coated beads was also

892   analyzed for these factors (Tm Expt 1 and Expt 2). Statistical significance (*p* values) was

893   calculated using a two-tailed Student's *t* test.

894

895   **Fig 4. P-TEFb biogenesis in primary T cells does not require intracellular mobilization of**

896   **calcium.** *(A) Left graph*; Cross-comparison of the effectiveness of PKC agonists on their own or

897   combined with ionomycin at inducing active P-TEFb in primary T cells measured by co-staining

898   for CyclinT1 and pSer175 CDK9. *Middle graph*; Cross-comparison of the effectiveness of PKC

899   agonists on their own or combined with ionomycin at coordinately inducing the C-terminal domain

900   Ser2 phosphorylated form of RNA polymerase II (pSer2 RNAP II CTD) and the transcriptionally

901   active form of NF-κB (pSer529 p65). *Right graph*; Measurement of the effect of PKC agonists on

902   their own or combined with ionomycin on cell viability as assessed by flow cytometry following

903   staining with the eFluor450 viability dye. *(B)* Coordinate reactivation of P-TEFb *(left graph)* and

904   latent HIV *(right graph)* upon 24 h treatment of resting Th17 cells with either 50 nM Ingenol or 1

37

905   µM Prostratin. The graphs show data generated using cells derived from at least three different

906   donors. *(C)* Proposed schemes for the promoter recruitment of RNA polymerase II (RNAP II) to

907   proviral HIV and its phosphorylation by active P-TEFb on its C-terminal domain (pSer2 RNAP II

908   CTD). Arrows with red question marks indicate a P-TEFb regulatory signaling pathway(s)

909   intended to be defined by the current study.

910

911   **Fig 5. Coordinate reactivation of P-TEFb and latent HIV in primary T cells is largely**

912   **independent of PKC activity.** *(A)* The PKC-θ inhibitor sotrastaurin is ineffective at repressing

913   ingenol- and prostratin-induced expression of P-TEFb but effective at blocking the activating

914   phosphorylation of the p65 subunit of NF-κB at Ser529 in response to these phorbol esters. CD4$^+$

915   memory T cells from four different donors were treated for 24 h with the indicated concentrations

916   of either ingenol or prostratin in the presence or absence of 100 nM sotrastaurin prior to subjecting

917   cells to dual immunofluorescence staining for CycT1 and pSer175 CDK9 *(top graph)* or

918   immunofluorescence staining for pSer529 p65 *(bottom graph)*. *(B)* PKC inhibitors modestly

919   repress TCR-mediated expression of P-TEFb in CD4$^+$ memory T cells but are unable to block

920   reactivation of proviral HIV in primary Th17 cells. *Left graph*; Measurement of P-TEFb expression

921   by dual CyclinT1 and pSer175 CDK9 immunofluorescence staining of memory T cells from four

922   different healthy donors that were treated or not with 100 nM Sotrastaurin for 30 min prior to TCR

923   co-stimulation anti-CD3/anti-CD28 Dynabeads for 24 h. *Right graph*; Assessment of the effect of

924   PKC inhibition on proviral reactivation in *ex vivo* HIV-infected primary Th17 cells prepared from

925   three healthy donors. Latently infected cells were treated or not for 30 min with 100 nM Ro-31-

926   8220 in two experiments or with 100 nM Sotrastaurin in three experiments prior to TCR co-

927   stimulation with anti-CD3/anti-CD28 Dynabeads for 24 h. Thereafter, cells were analyzed by flow

928   cytometry following immunostaining using a fluorophore-conjugated antibody towards HIV Nef.

929   Statistical significance (*p* values) was calculated using a two-tailed Student's *t* test. *(C)* PKC

930   inhibitors are unable to repress the reactivation of latent HIV in primary Th17 cells in response to

931   PKC agonists. Latently infected Th17 cells were treated or not for 30 min with either a combination

932   of Ro-31-8220 and Gö 6983 (Donor 14 (Expt 1) and Donor 14 (Expt 2)) at 100 nM each or 100

933   nM Sotrastaurin (Donor 16 (Expt 1) and Donor 14 (Expt 3)) prior to TCR co-stimulation or

934   challenge with the PKC agonists shown. Thereafter, cells were immunostained using a

935   fluorophore-conjugated antibody towards HIV Nef and analyzed by flow cytometry.

936

937   **Fig 6. Inducible P-TEFb expression and proviral reactivation in primary T cells are**

938   **mediated by the MAPK ERK pathway.** *(A)* Proposed scheme for the intracellular stimulation of

939   P-TEFb expression in primary T cells by PKC agonists or TCR-generated diacylglycerol (DAG)

940   leading up to the reactivation of HIV from latency. *(B)* The MEK inhibitor U0126 substantially

941   blocks expression of active P-TEFb in primary CD4^{+} T cells in response to treatment with PKC

942   agonists but modestly inhibits P-TEFb expression in response to TCR co-stimulation. Cells were

943   treated or not for 30 min with U0126 at the indicated concentrations prior to TCR co-stimulation

944   with anti-CD3/anti-CD28 Dynabeads or challenge with either 50 nM Ingenol or 1 μM Prostratin for

945   24 h. Cells were then analyzed by flow cytometry for P-TEFb expression following immunostaining

946   using fluorophore-conjugated antibodies towards CyclinT1 and pSer175 CDK9. *(C)* U0126

947   substantially retards proviral reactivation in primary Th17 cells in response to TCR co-stimulation

948   or challenge with PKC agonists. Latently infected Th17 cells prepared using naïve CD4^{+} T cells

949   from two different donors were pretreated or not with 20 μM U0126 for 30 min prior to TCR co-

950   stimulation with anti-CD3/anti-CD28 Dynabeads or challenge with 50 nM ingenol or 1 μM

951   prostratin for 24 h. Cells were analyzed by flow cytometry following immunostaining using a

952   fluorophore-conjugated antibody towards HIV Nef. All the *p* values shown in *B* and *C* were

953   calculated using a two-tailed Student's *t* test.

954

955 **Fig 7. Memory CD4^{+} T cells predominantly express the RasGRP1 isoform which rapidly**

956 **localizes to the plasma membrane upon phorbol ester stimulation.** *(A)* Immunofluorescence

957 microscopy of healthy donor memory CD4^{+} T cells with or without a 2-h stimulation with TCR

958 Dynabeads, 50 nM ingenol, or 1 μM prostratin. Following stimulation cells were fixed in 4%

959 formaldehyde, permeabilized, and then immunofluorescently co-stained for RasGRP1 and a

960 constitutively expressed membrane protein Ezrin. After counterstaining with DAPI, coverslips

961 were mounted on to glass slides and imaged using a DeltaVision deconvolution microscope at

962 100X. Scale bar represents a length of 5 μm. *(B)* Degree of membrane colocalization of RasGRP1

963 and Ezrin as assessed by measuring multiple Pearson's correlation coefficient values obtained

964 from defined regions of interest. Selected regions of interest defined as single cells were analyzed

965 using the softWoRx colocalization module to measure the degree of colocalization between

966 fluorescently stained RasGRP1 and Ezrin. Statistical significance (*p* values) was calculated using

967 a two-tailed Student's *t* test. *(C)* UMAP clustering based on scRNA-seq profiling of resting

968 unstimulated and TCR-activated memory CD4^{+} T cells. *(D)* Assessment of the expression profiles

969 of RasGRP isoforms in memory CD4^{+} T cells by scRNA Drop-seq.

970

971 **Fig 8. Inhibition of PI3K and mTORC kinases partially suppresses TCR-induced P-TEFb**

972 **expression and modestly inhibits TCR-induced proviral reactivation in primary CD4^{+} T**

973 **cells.** *(A)* Proposed role of the PI3K/AKT/mTOR signaling pathway in mediating the biogenesis

974 of P-TEFb. Inhibitors that were tested in the current study are shown in black italics. *(B)* PI3K and

975 mTORC kinase inhibitors partially repress TCR-induced expression of active P-TEFb and albeit

976 with significant variability. Memory CD4^{+} T cells (three experiments) and resting primary Th17

977 cells (four experiments) were treated or not for 30 min with inhibitors targeting PI3K (LY294002),

978 mTORC1 (Rapamycin) or both mTORC1 and mTORC2 (Torin) at the concentrations shown prior

979 to TCR co-stimulation for 24 h. Thereafter, cells were examined for active P-TEFb expression by

980 flow cytometry following dual immunofluorescence staining for cyclinT1 and pSer175 CDK9. *(C)*

981 PI3K and mTORC kinase inhibitors modestly repress proviral reactivation that is in response to

982 TCR co-stimulation. Latently infected Th17 cells prepared using naïve CD4⁺ T cells isolated from

983 three healthy donors were pretreated or not for 30 min with inhibitors to PI3K (LY294002) or

984 mTORC1/mTORC2 (Torin) prior to stimulating the cells through the TCR for 24 h. Cells were

985 thereafter analyzed by flow cytometry following immunostaining using a fluorophore-conjugated

986 antibody towards HIV Nef. Statistical significance (*p* values) in *B* and *C* was determined using a

987 two-tailed Student's *t* test.

988

989 **Fig 9. Combined inhibition of MEK and mTORC kinases abrogates TCR-induced P-TEFb**

990 **expression and effectively suppresses TCR-mediated proviral reactivation in primary CD4⁺**

991 **T cells.** *(A)* Combined inhibition of MEK and PI3K kinases enhances the disruption of the active

992 P-TEFb expression. Healthy donor memory CD4⁺ T cells (in two of the plotted experiments) and

993 polarized resting primary Th17 cells (in two of the plotted experiments) were treated or not for 30

994 min with inhibitors targeting either MEK (U0126) or PI3K (LY294002) or both at the concentrations

995 shown prior to TCR co-stimulation for 24 h. Thereafter, cells were examined for active P-TEFb

996 expression by flow cytometry following dual immunofluorescence staining for cyclinT1 and

997 pSer175 CDK9. Cell viability was also assessed by flow cytometry in three of the four experiments

998 following staining with the eFluor450 viability dye. The viability data shown are expressed as a

999 percentage relative to non-treated cells. *(B)* Combined inhibition of MEK and mTORC abrogates

1000 the expression of active P-TEFb. Memory CD4⁺ T cells (four experiments) and resting primary

1001 Th17 cells (two experiments) were treated or not for 30 min with the MEK inhibitor U0126 on its

1002 own or in combination with inhibitors targeting mTORC1 (Rapamycin) or both mTORC1 and

1003 mTORC2 (Torin) at the concentrations shown prior to TCR co-stimulation for 24 h. Thereafter,

1004 cells were examined for active P-TEFb expression by flow cytometry following dual

1005 immunofluorescence staining for cyclinT1 and pSer175 CDK9. In four of the experiments shown,

41

1006   cell viability was assessed by flow cytometry following staining with the eFluor450 viability dye.

1007   The viability data are expressed as a percentage relative to non-treated cells. Statistical

1008   significance (*p* values) in *A* and *B* was determined using a two-tailed Student's *t* test. *(C)* Latently

1009   infected Th17 cells were pretreated or not for 30 min with the MEK inhibitor U0126 or the

1010   mTORC1/2 inhibitor Torin on their own or in combination prior to TCR co-stimulation for 24 h.

1011   Afterwards, cells were analyzed by flow cytometry following immunostaining using a fluorophore-

1012   conjugated antibody towards HIV Nef.

1013

1014   **Fig 10. Model for the reactivation of P-TEFb and proviral HIV by RasGRP1-Ras-Raf-MEK-**

1015   **ERK1/2 and PI3K-AKT-mTORC1/2 signaling in latently infected CD4$^+$ T cells.** Generation of

1016   diacylglycerol (DAG) by activated PLC-γ1 immediately following TCR co-stimulation enables the

1017   recruitment of RasGRP1 to the plasma membrane, the initial activation of Ras via GTP loading

1018   and the allosteric activation of membrane-anchored SOS by GTP-bound Ras. This can also be

1019   accomplished by DAG-mimicking PKC agonists. Allosteric activation of SOS creates a positive

1020   Ras-GTP-SOS feedback loop that leads to maximal activation of Ras and formation of Ras-Raf

1021   complexes at the membrane. A phosphorylation cascade is triggered by Ras-Raf leading to

1022   activation of ERK1 and ERK2 which is turn stimulate the biogenesis of P-TEFb via mechanisms

1023   that are yet to be defined. RasGRP1 activity may be regulated by controlling the availability of

1024   DAG which can get converted to phosphatidic acid (PA) primarily by DGK-α. TCR co-stimulation

1025   also rapidly stimulates PI3K leading to the activation of mTORC1 which may regulate P-TEFb

1026   biogenesis by enabling posttranscriptional synthesis of cyclin T1 (CycT1). Inhibitor-based

1027   experiments have clarified that intracellular calcium release and the activation of PKC are

1028   dispensable for formation of transcriptionally active P-TEFb. However, the recruitment of RNA

1029   polymerase II (RNAP II) to proviral HIV and its eventual phosphorylation by P-TEFb to stimulate

1030   processive transcription elongation are likely to be dependent on both Ca^{++} and PKC signaling.

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1439

Supporting information

1440

1441 **S1 Fig. Isolation of memory CD4⁺ T cells and their characterization.** *(A)* Surface phenotype

1442 of memory CD4⁺ T cells isolated using the EasySep™ Memory CD4⁺ T Cell Enrichment Kit (Cat.

1443 # 19165). Following memory CD4⁺ T-cell isolation cells were treated or not for 24 h with the stimuli

1444 shown then subjected to immunofluorescence flow cytometry after staining with fluorophore-

1445 conjugated isotype controls or antibodies towards CD25 and CD45-RO. (B) Central memory CD4⁺

1446 T cells account for the majority of both total peripheral CD4⁺ and memory CD4⁺ T cells. Total

1447 peripheral CD4+ T cells were isolated from healthy donor PBMCs using EasySep™ Human CD4⁺

1448 T Cell Kit (Cat. # 17952). Thereafter, cells were subjected to immunofluorescence flow cytometry

1449 after staining with fluorophore-conjugated antibodies towards CCR7 and CD45-RO. Based on this

1450 dual staining, CD4⁺ T-cell subsets were determined in multiple experiments as a fraction of total

1451 peripheral CD4⁺ or total memory CD4⁺ T cells.

1452

1453 **S2 Fig. Early expression of P-TEFb coincides with the appearance of T-cell activation and**

1454 **proliferative markers in memory CD4⁺ T cells.** Untreated and 4 h or 24 h TCR-activated

1455 memory CD4⁺ T cells were subjected to immunofluorescence flow cytometry to monitor the

1456 expression of cyclinT1, pSer175 or pThr186 T-loop phosphorylated CDK9, the activated form of

1457 NF-κB (pSer529 p65 NF-κB), T-cell proliferative markers Ki67 and CyclinD3, and the T-cell

1458 activation surface markers CD25 and CD69.

1459

1460 **S3 Fig. Kinetic examination of P-TEFb, Ki67 and cyclinD3 expression in central and effector**

1461 **memory CD4⁺ T-cell subsets following TCR co-stimulation.** *(A)* and *(B)* Following purification

1462 of these memory subsets from healthy donor PBMCs using the EasySep™ Human Central and

1463 Effector Memory CD4⁺ T Cell Isolation Kit (Cat. # 17865), they were stimulated through the TCR

1464   with anti-CD3/anti-CD28 Dynabeads for varying times as shown. Thereafter, cells were subjected

1465   to immunofluorescence flow cytometry to monitor the expression of the P-TEFb (pSer175 CDK9

1466   and cyclinT1), Ki67 and cyclin D3.

1467

1468   **S4 Fig. PKC agonists can sufficiently stimulate active P-TEFb expression in polarized**

1469   **primary Th17 cells.** *(A)* Procedure for generating polarized quiescent primary Th17 cells from

1470   naïve CD4$^+$ T cells with or without a latent infection with HIV. *(B)* A representative

1471   immunofluorescence flow cytometry experiment showing that PKC agonists can sufficiently

1472   induce active P-TEFb expression in polarized primary Th17 cells while TNF-α and the HDAC

1473   inhibitor SAHA are poor stimulators. Active P-TEFb expression was assessed by examining the

1474   dual expression of cyclinT1 and pSer175 CDK9 using fluorophore-conjugated antibodies.

1475

1476   **S5 Fig. Reactivation of latent HIV in primary Th17 cells by TCR co-stimulation or PKC**

1477   **agonists is unlikely to be mediated by PKC.** *(A)* Rapid expression of active P-TEFb in memory

1478   CD4$^+$ T cells in response to TCR co-stimulation. Memory T cells from three different healthy

1479   donors (color-coded) were activated or not for 2 h through the TCR with anti-CD3/anti-CD28

1480   Dynabeads. Afterwards, the cells were subjected to flow cytometry analysis following intracellular

1481   immunofluorescence staining for P-TEFb (by co-staining for CycT1 and pSer175 CDK9) or the C-

1482   terminal domain Ser2 phosphorylated form of RNA polymerase II (pSer2 RNAP II CTD). *(B)* A

1483   selective inhibitor of CDK9 kinase, flavopiridol (FVP) effectively blocks TCR-mediated proviral

1484   reactivation in the QUECEL primary Th17 model of HIV latency. Latently infected Th17 cells were

1485   pretreated or not with 100 nM FVP for 30 min prior to TCR co-stimulation with anti-CD3/anti-CD28

1486   Dynabeads for 24 h. Thereafter, cells were analyzed by flow cytometry following immunostaining

1487   using a fluorophore-conjugated antibody towards the HIV Nef accessory protein. Error bars

1488   denote S.E. of the mean from three separate experiments. Statistical significance (*p* values) in

1489   both *A* and *B* was calculated using a two-tailed Student's *t* test. *(C)* TNF-α and SAHA are

60

1490   inadequately able to reactivate latent HIV in primary Th17 cells. Proviral HIV expression was

1491   examined by immunofluorescence flow cytometry following immunostaining using a fluorophore-

1492   conjugated antibody towards the HIV Nef accessory protein. *(D)* Ingenol, prostratin and PMA can

1493   sufficiently reactivate latent HIV in primary Th17 cells. *(E)* Representative flow cytometry data

1494   showing that a combination of two PKC inhibitors (Ro-31-8220 and Gö 6983) are ineffective at

1495   blocking proviral reactivation in response to treatment with 200 nM ingenol or 1 μM prostratin. *(F)*

1496   Viability assessment of cells treated or not with a combination of Ro-31-8220 and Gö 6983 at 100

1497   nM each prior to TCR co-stimulation or challenge with PKC agonists. Cell viability was assessed

1498   by flow cytometry following staining with the eFluor450 viability dye. The viability data shown are

1499   expressed as a percentage relative to non-treated cells.

1500

1501   **S6 Fig. Immunoblotting and bulk RNA-seq analysis of P-TEFb, 7SK snRNP and SEC**

1502   **expression in primary CD4$^+$ T cells.** *(A)* Immunoblotting analysis of resting and TCR-activated

1503   primary Th17 cells to examine the expression of P-TEFb subunits (CDK9 and CycT1), its 7SK

1504   snRNP inhibitory partner HEXIM1, SEC components (ELL2, AFF1 and ENL), and transcription

1505   factors (NF-κB, NFAT-c1 and AP1). Resting polarized Th17 cells prepared from healthy donor

1506   naïve CD4$^+$ T cells were stimulated or not with anti-CD3/anti-CD28 Dynabeads for the times

1507   shown prior to preparation of low salt and chromatin nuclear fractions. Thereafter, both fractions

1508   were subjected to Western blotting using primary antibodies towards the proteins shown. *(B)*, *(C)*

1509   and *(D)* Examination of the mRNA expression of P-TEFb, SEC and 7SK snRNP subunits using a

1510   publicly available bulk RNA-seq dataset (SRA accession SRP026389) prepared using human

1511   memory CD4$^+$ T cells that had been activated or not through TCR co-stimulation with anti-

1512   CD3/anti-CD28 coated beads for the times shown.

1513

1514 **S7 Fig. Single-cell transcriptome profiling of the expression of P-TEFb, SEC and 7SK**

1515 **snRNP subunits in memory CD4⁺ T cells.** *(A)* and *(B)* Healthy donor-derived memory CD4⁺ T

1516 cells were allowed to recover overnight in complete RPMI medium with no IL-2 addition prior to

1517 challenging them or not with anti-CD3/anti-CD28 Dynabeads for 24 or 72 h at a 1:1 bead-to-cell

1518 ratio in media containing 15 IU/ml IL-2. Thereafter, cells were prepared for Drop-seq by

1519 magnetically removing the T-cell receptor activator beads, removal of dead cells using the MACS

1520 dead cell removal kit and resuspension of cells in 1X PBS containing 0.1% BSA at 2 X 10^5 cells/ml.

1521 Cells were clustered according to their unbiased transcriptome signatures (unsupervised

1522 clustering) and the dot plot function in Seurat was used to quantitate positive cell enrichment and

1523 relative expression levels of P-TEFb, SEC and 7SK snRNP transcripts in the dataset.

1524

1525 **S8 Fig. Pan-PKC inhibitors are ineffective at suppressing activation loop phosphorylation**

1526 **of CDK9 at Ser175 and Thr186 in memory CD4⁺ T cells.** Cells were treated or not for 30 min

1527 with a combination of Ro-31-8220 and Gö 6983 at 100 nM each prior to stimulation with anti-

1528 CD3/anti-CD28 Dynabeads or challenge with ingenol or prostratin. Afterwards, cells were

1529 analyzed by flow cytometry following immunostaining using fluorophore-conjugated antibodies

1530 towards pThr186 CDK9 and pSer175 CDK9.

1531

1532 **S9 Fig. Elevation of active P-TEFb expression in memory CD4⁺ T cells following TCR co-**

1533 **stimulation is not dependent on PKC activity.** *(A)* Scheme for the activation of the transcription

1534 factor NF-κB by PKC in primary T cells upon TCR co-stimulation. *(B)* Immunoblotting analysis of

1535 memory CD4⁺ T-cell extracts demonstrating that the pan-PKC inhibitor Ro-31-8220 can retard the

1536 degradation on IκB-α and the nuclear induction of NF-κB. Healthy donor memory CD4⁺ T cells

1537 were treated or not with 100 nM Ro-31-8220 for 30 min prior to stimulation with anti-CD3/anti-

1538 CD28 Dynabeads for the times shown. Thereafter, low salt and chromatin nuclear fractions were

1539 prepared and subjected to Western blotting using primary antibodies towards IκB-α and NF-κB

1540 subunits p50 and p65. *(C)* PKC inhibitors are unable to suppress the induction of active P-TEFb

1541 in memory CD4^{+} T cells. Cells were treated or not for 30 min with Ro-31-8220, Gö 6950, or Gö

1542 6983 at 100 nM each prior to TCR co-stimulation. Thereafter, cells were analyzed by flow

1543 cytometry following immunostaining using fluorophore-conjugated antibodies towards cyclinT1

1544 and pSer175 CDK9.

1545

1546 **S10 Fig. The MEK inhibitor U0126 effectively blocks the activation of ERK1/2 and the**

1547 **nuclear expression of c-Fos in T cells.** *(A)* Kinetic analysis of the activation of the MAP kinase

1548 isoforms ERK1 and ERK2, and the nuclear mobilization of c-Fos and NF-κB in response to PMA

1549 treatment with or without pretreatment with the MEK inhibitor U0126. Cytosolic and nuclear

1550 extracts were prepared from Jurkat T cells that were challenged or not with PMA for the indicated

1551 times in the presence or absence of U0126. These extracts were subjected to Western blotting to

1552 examine the expression of ERK1/2 and their phosphorylation by MEK, inducible expression of the

1553 transcription factor c-Fos, and the nuclear induction of c-Fos, NF-κB and NFAT proteins. *(B)* TCR

1554 activation of ERK1/2 phosphorylation by MEK in memory CD4^{+} T cells and subsequent ERK

1555 regulation of c-Fos are effectively blocked by U0126. Whole cell extracts were prepared from

1556 healthy donor memory CD4^{+} T cells stimulated or not through the TCR with anti-CD3/anti-CD28

1557 Dynabeads for the times shown. These extracts were subjected to Western blotting to examine

1558 the expression of ERK1/2 and their phosphorylation by MEK, and inducible expression of the

1559 transcription factor c-Fos.

1560

1561 **S11 Fig. MEK inhibitor U0126 effectively blocks the formation of active P-TEFb in memory**

1562 **CD4^{+} T cells that is in response to stimulation with PKC agonists.** Healthy donor memory

1563 CD4^{+} T cells were treated or not for 30 min with U0126 prior to 24 h TCR co-stimulation or

1564 challenge with ingenol, prostratin or PMA. Afterwards, cells were analyzed by flow cytometry for

1565 active P-TEFb expression following immunostaining using fluorophore-conjugated antibodies

1566 towards cyclinT1 and pSer175 CDK9.

1567

1568 **S12 Fig. MEK inhibitor U0126 effectively blocks synthesis of cyclin T1 and formation of**

1569 **pThr186 CDK9 in memory CD4$^+$ T cells in response to PKC agonists.** Healthy donor memory

1570 CD4$^+$ T cells were treated or not for 30 min with U0126 prior to 24 h TCR co-stimulation or

1571 challenge with ingenol or PMA. Afterwards, cells were analyzed by flow cytometry for cyclin T1

1572 and pThr186 CDK9 expression following immunostaining using fluorophore-conjugated

1573 antibodies towards cyclin T1 and pThr186 CDK9.

1574

1575 **S13 Fig. MEK inhibitor U0126 effectively blocks the formation of active P-TEFb in polarized**

1576 **primary Th17 cells that is in response to stimulation with PKC agonists.** Resting primary

1577 Th17 cells were treated or not for 30 min with U0126 prior to 24 h TCR co-stimulation or challenge

1578 with ingenol, prostratin or PMA. Afterwards, cells were analyzed by flow cytometry for active P-

1579 TEFb expression following immunostaining using fluorophore-conjugated antibodies towards

1580 cyclinT1 and pSer175 CDK9.

1581

1582 **S14 Fig. Representative experiment demonstrating the effect of MEK inhibitor U0126 on**

1583 **proviral reactivation in polarized primary Th17 cells in response to TCR-costimulation and**

1584 **PKC agonists.** Latently infected resting primary Th17 cells were treated or not for 30 min with

1585 U0126 prior to 24 h TCR co-stimulation or challenge with the PKC agonists ingenol, prostratin or

1586 PMA. Thereafter, cells were analyzed by flow cytometry following immunostaining using a

1587 fluorophore-conjugated antibody towards HIV Nef.

1588

1589 **S15 Fig. Effect of MEK and JNK inhibition on proviral expression in primary T cells.** *(A)*

1590 Proposed scheme for the regulation of proviral reactivation by ERK and JNK MAPK signaling in

1591 TCR-activated primary T cells. *(B)* U0126 performs better than the JNK inhibitor SP600125 at

1592 suppressing the reactivation of latent HIV by TCR co-stimulation in HIV-infected Th17 cells albeit

1593 with noticeably significant variability. Latently infected primary Th17 cells prepared using naïve

1594 CD4⁺ T cells from three different donors were pretreated or not with U0126 or SP600125 for 30

1595 min prior to TCR co-stimulation with anti-CD3/anti-CD28 Dynabeads for 24 h. Cells were then

1596 analyzed by flow cytometry following immunostaining using a fluorophore-conjugated antibody

1597 towards HIV Nef. The *p* values shown were calculated using a two-tailed Student's *t* test.

1598

1599 **S16 Fig. The MAPK ERK pathway is a primary mediator of proviral reactivation in TCR-**

1600 **activated primary T cells.** *(A)* Latently infected Th17 cells prepared using healthy donor naïve

1601 CD4⁺ T cells were treated or not for 30 min with either the MEK inhibitor U0126 or the JNK inhibitor

1602 SP600125 on their own or in combination prior to 24 h TCR co-stimulation or challenge with either

1603 ingenol or prostratin. Afterwards, cells were analyzed by flow cytometry following immunostaining

1604 using a fluorophore-conjugated antibody towards HIV Nef. The graph shows data from two

1605 separate experiments. (B) Viability assessment by propidium iodide staining of memory CD4⁺ T

1606 cells treated with U0126 or SP600125 on their own or in combination prior to 24 h TCR co-

1607 stimulation or challenge with either ingenol or prostratin. The viability data shown are expressed

1608 as a percentage relative to non-treated cells.

1609

1610 **S17 Fig. MAPK ERK is the primary MAP kinase that mediates phorbol ester-induced P-**

1611 **TEFb expression in memory CD4⁺ T cells.** Healthy donor memory CD4⁺ T cells were treated or

1612 not for 30 min with either the MEK inhibitor U0126 or the JNK inhibitor SP600125 on their own or

1613 in combination prior to 24 h TCR co-stimulation or challenge with ingenol. Afterwards, cells were

1614 analyzed by flow cytometry for active P-TEFb expression following immunostaining using

1615 fluorophore-conjugated antibodies towards cyclin T1 and pSer175 CDK9.

1616

1617 **S18 Fig. A Raf inhibitor AZ 628 effectively blocks the formation of active P-TEFb in memory**

1618 **CD4⁺ T cells that is in response to stimulation with PKC agonists.** Healthy donor memory

1619 CD4⁺ T cells were treated or not for 30 min with AZ 628 prior to 24 h TCR co-stimulation or

1620 challenge with ingenol or prostratin. Afterwards, cells were analyzed by flow cytometry for active

1621 P-TEFb expression following immunostaining using fluorophore-conjugated antibodies towards

1622 cyclinT1 and pSer175 CDK9.

1623

1624 **S19 Fig. Bulk RNA-seq analysis of the expression of RasGRP, Ras, Raf and Rap1 isoforms**

1625 **in primary QUECEL subsets and memory CD4⁺ T cells.** Quiescent CD4⁺ T cells that had been

1626 polarized into Th1, Th2, Treg and Th17 subsets using the QUECEL procedure were activated or

1627 not for 24 h with anti-CD3/anti-CD28 Dynabeads. Bulk RNA-seq datasets obtained using these

1628 cells were analyzed to examine the expression of RasGRP *(A)*, Ras *(C)*, Raf *(D)* and Rap1 *(E)*

1629 isoforms. A publicly available bulk RNA-seq dataset of primary human memory CD4⁺ T cells that

1630 had been activated or not through TCR co-stimulation with anti-CD3/anti-CD28 coated beads

1631 (SRA accession SRP026389) was also analyzed for these factors (Tm Expt 1 and Expt 2). In *B*,

1632 additional assessment of RasGRP isoform expression was performed over the entire time course

1633 of TCR activation (0, 2, 4, 6, and 24 h) for which the memory T-cell bulk RNA-seq dataset was

1634 generated. Transcripts per million (TPM) values were used to evaluate the relative abundance of

1635 transcripts under resting and TCR-activated conditions. Statistical significance (*p* values) was

1636 calculated using a two-tailed Student's *t* test.

1637

1638 **S20 Fig. Transcriptome profiling of PKC, DGK and mTORC transcripts in primary QUECEL**

1639 **subsets and memory CD4⁺ T cells.** *(A)* scRNA-seq analysis of unstimulated and TCR-activated

1640 memory CD4⁺ T cells. Healthy donor-derived memory CD4+ T cells were stimulated or not with

1641 anti-CD3/anti-CD28 Dynabeads for 24 or 72 h prior to being subjected to Drop-seq. Cells were

1642 clustered according to their unbiased transcriptome signatures (unsupervised clustering) and the

1643 dot plot function in Seurat was used to quantitate positive cell enrichment and relative expression

1644 levels of PKC, DGK and mTORC transcripts in the dataset. *(B)* Bulk RNA-seq analysis of primary

1645 QUECEL subsets and memory CD4+ T cells. Quiescent CD4+ T cells that had been polarized into

1646 Th1, Th2, Treg and Th17 subsets using the QUECEL procedure were activated or not for 24 h

1647 with anti-CD3/anti-CD28 Dynabeads. Bulk RNA-seq datasets obtained using these cells were

1648 analyzed to examine the expression of PKC, DGK and mTORC transcripts. A publicly available

1649 bulk RNA-seq dataset of primary human memory CD4+ T cells that had been activated or not

1650 through TCR co-stimulation with anti-CD3/anti-CD28 coated beads (SRA accession SRP026389)

1651 was also included in the analysis (Tm Expt 1 and Expt 2).

1652

1653 **S21 Fig. Effect of inhibiting AKT, GSK3β and PTEN on TCR-induced P-TEFb expression in**

1654 **memory CD4+ T cells.** *(A)* and *(B)* Healthy donor memory CD4+ T cells were treated or not for

1655 30 min with inhibitors towards AKT (Ipatasertib), PTEN (VO-OHpic), GSK3β (SB216763 or

1656 Tideglusib) or PI3K (LY294002) at the concentrations shown prior 24 h TCR co-stimulation.

1657 Afterwards, cells were analyzed by flow cytometry for active P-TEFb expression following

1658 immunostaining using fluorophore-conjugated antibodies towards cyclinT1 and pSer175 CDK9.

1659

1660 **S22 Fig. Representative flow cytometry experiment demonstrating the effect of treating**

1661 **polarized primary Th17 cells with U0126 or LY292004 on their own or in combination on**

1662 **TCR-induced P-TEFb expression.** Resting primary Th17 cells were treated or not for 30 min

1663 with inhibitors targeting either MEK (U0126) or PI3K (LY294002) or both at the concentrations

1664 shown prior to TCR co-stimulation for 24 h. Thereafter, cells were examined for active P-TEFb

1665 expression by flow cytometry following dual immunofluorescence staining for cyclinT1 and

1666 pSer175 CDK9.

1667

1668   **S23 Fig. Combined inhibition of MEK and mTORC kinases substantially suppresses TCR-**

1669   **mediated proviral reactivation in primary Th17 cells.** *(A)* Latently infected Th17 cells prepared

1670   using naïve CD4$^+$ T cells isolated from a healthy donor were pretreated or not for 30 min with the

1671   inhibitors shown prior to TCR co-stimulation for 24 h. Afterwards, cells were analyzed by flow

1672   cytometry following immunostaining using a fluorophore-conjugated antibody towards HIV Nef.

1673   *(B)* Assessment of the effect of combined inhibitor treatment on cell viability. Cell viability was

1674   assessed by flow cytometry following staining with the eFluor450 viability dye. The viability data

1675   shown are expressed as a percentage relative to non-treated cells.

1676

A — Scheme for P-TEFb biogenesis

B — Transcriptionally active P-TEFb

C — Subunit expression in primary T cells

D — Assessment of P-TEFb-inducing stimuli

◆ Memory CD4 T cells   ◆ Primary Th17 cells

Fig 1

A

Generation of P-TEFb in memory T cells

	No stim		No stim	4 h TCR	24 h TCR

CDK9 / **Cdc37** / **CDK9/Cdc37** (left panels)

CDK9 / **CycT1** / **CDK9/CycT1** (right panels)

B

7SK snRNP assembly

	No stim	2 h TCR	24 h TCR	24 h Prostratin + Ionomycin

7SK

HEXIM1

7SK/HEXIM1

Fig 2

A   P-TEFb and CD25

CycT1   CycT2   CDK9   CD25

B   7SK snRNP

HEXIM1   HEXIM2   LARP7   MEPCE

C   Super Elongation Complex

AFF1   AFF4   ELL   ELL2   ENL

◆Th17 ◆Th1 ◆Th2 ◆Treg ◆Tm (Expt 1) ◆Tm (Expt 2)

Fig 3

A PKC agonists stimulate P-TEFb expression with minimal toxicity
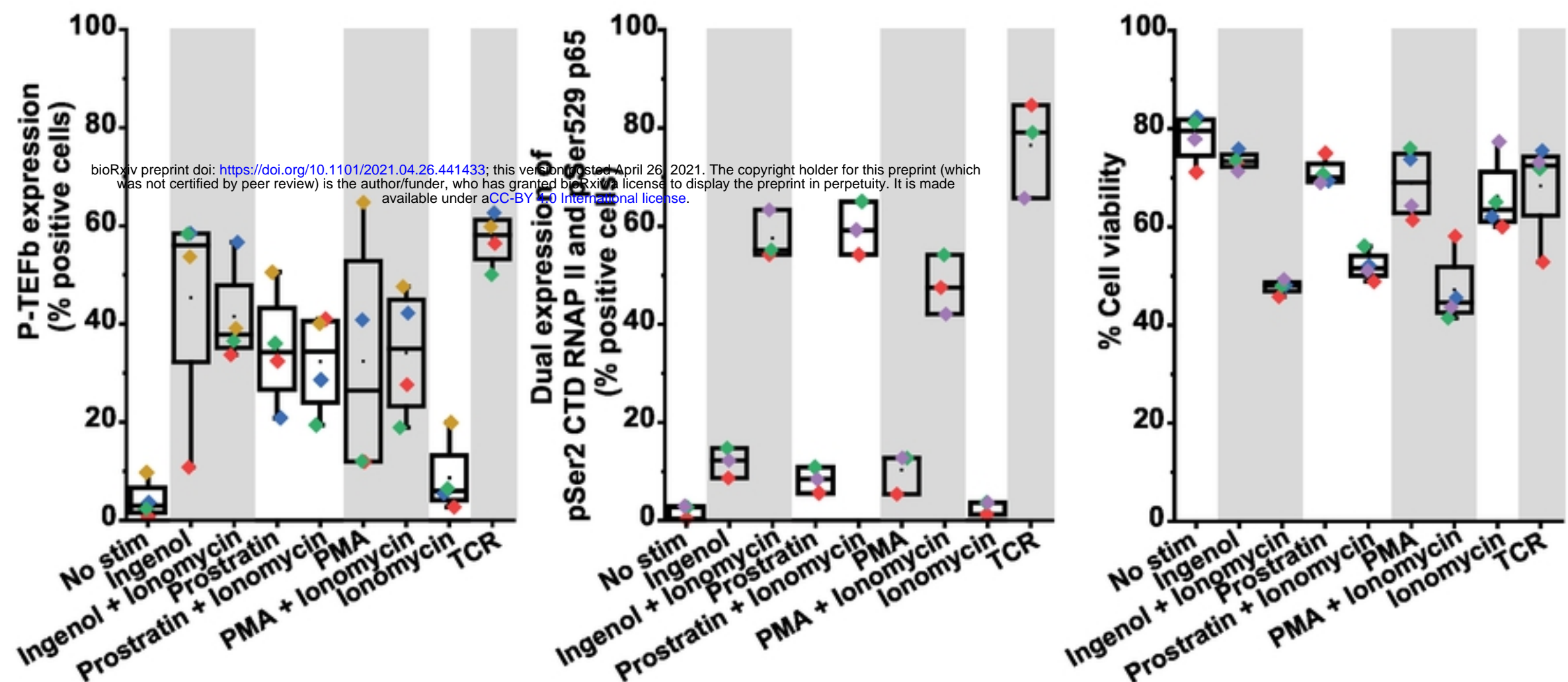
B Reactivation of HIV in QUECEL model
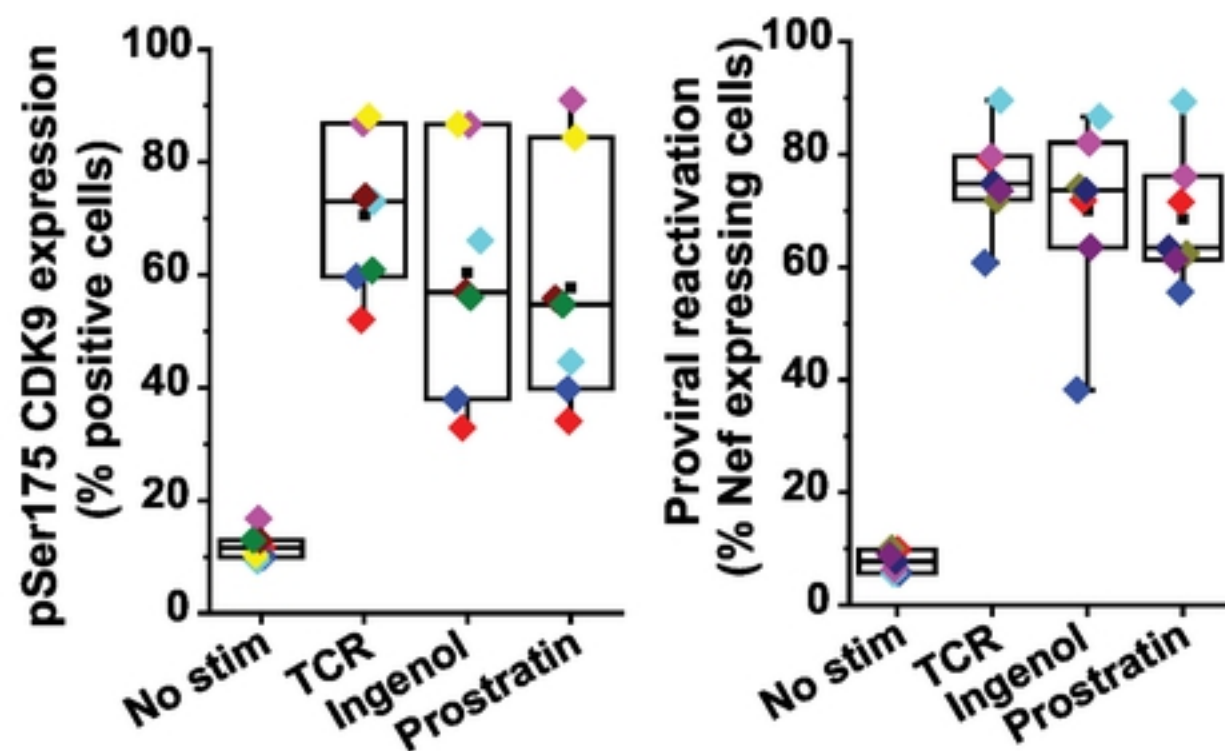
C Signals that generate P-TEFb
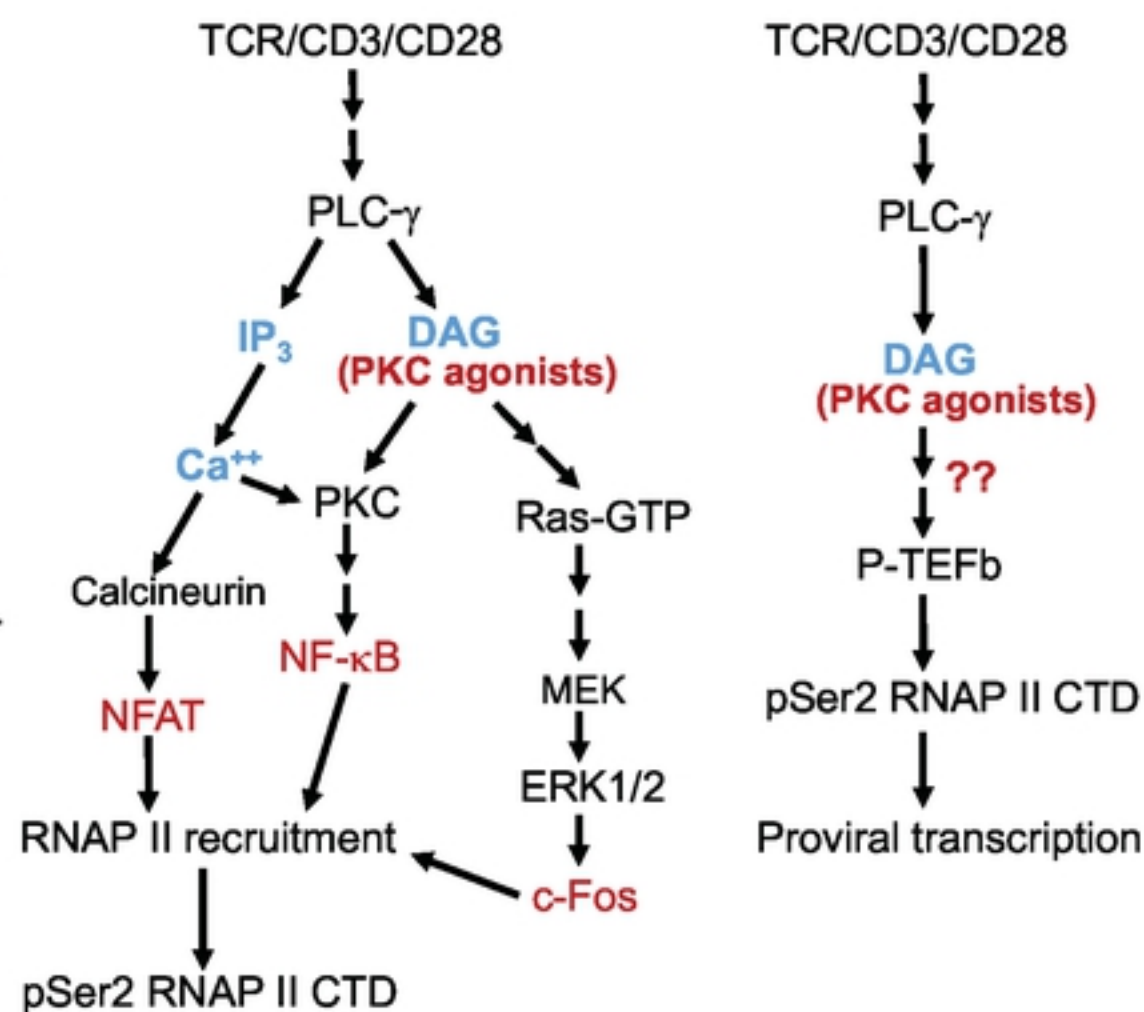
Fig 4

A PKC-θ inhibition fails to block P-TEFb biogenesis

B Pan-PKC inhibitors modestly retard P-TEFb and HIV reactivation

Memory CD4 T cells

Polarized Th17 cells

C PKC-θ and pan-PKC inhibition ineffectively block HIV reactivation
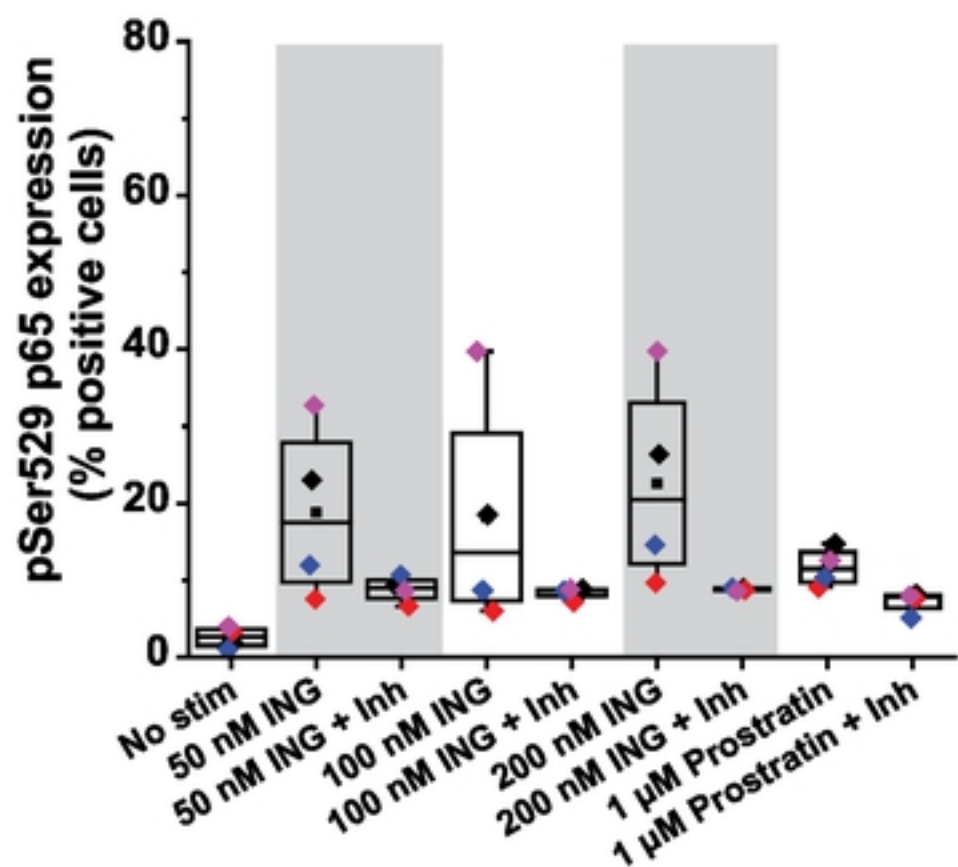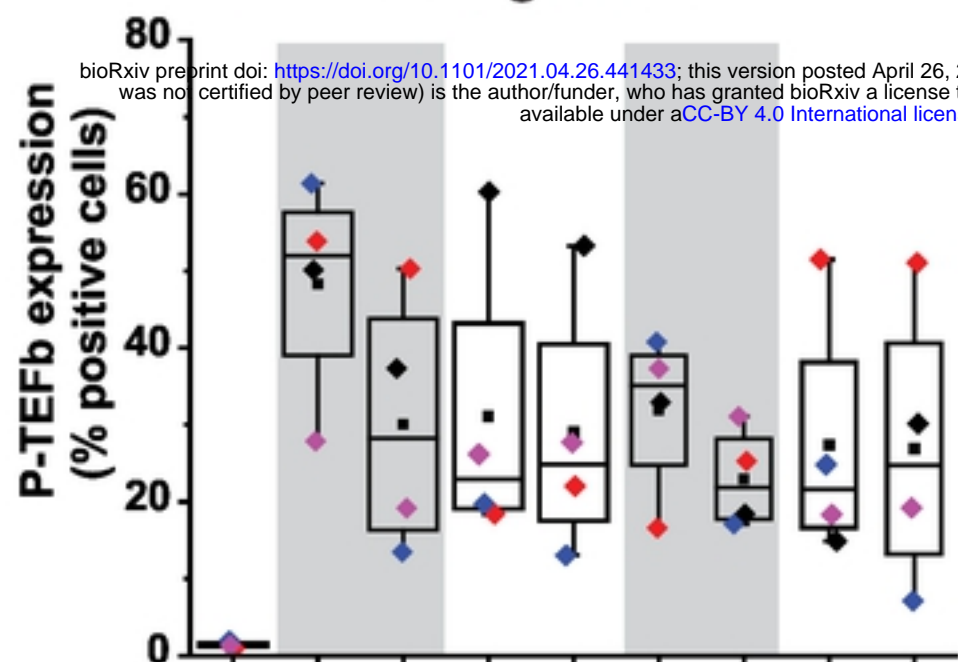
◆ Donor 9 ◆ Donor 10 ◆ Donor 15 ◆ Donor 16

◆ Donor 14 (Expt 1)
◆ Donor 14 (Expt 2)
◆ Donor 16 (Expt 1)
◆ Donor 14 (Expt 3)

Fig 5

A MAPK ERK signaling

B Effect of MEK inhibition on P-TEFb expression

C Effect of MEK inhibition on HIV reactivation
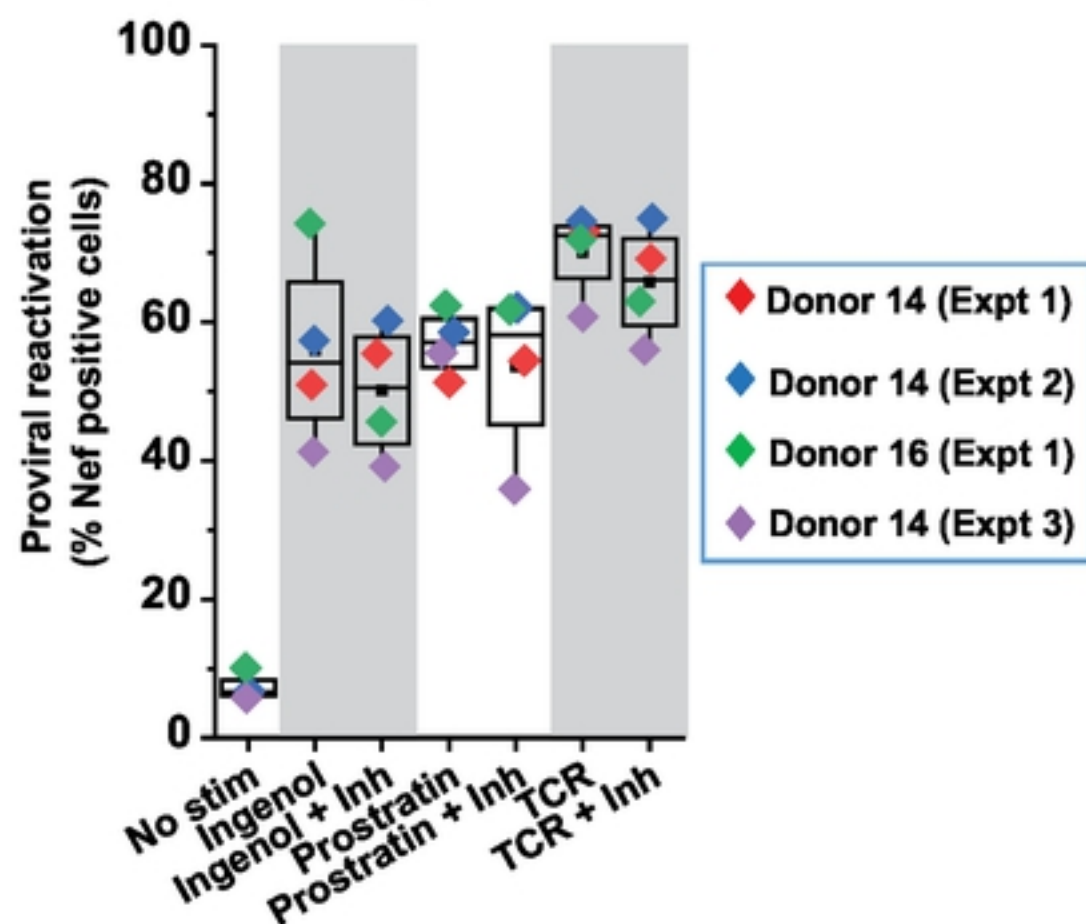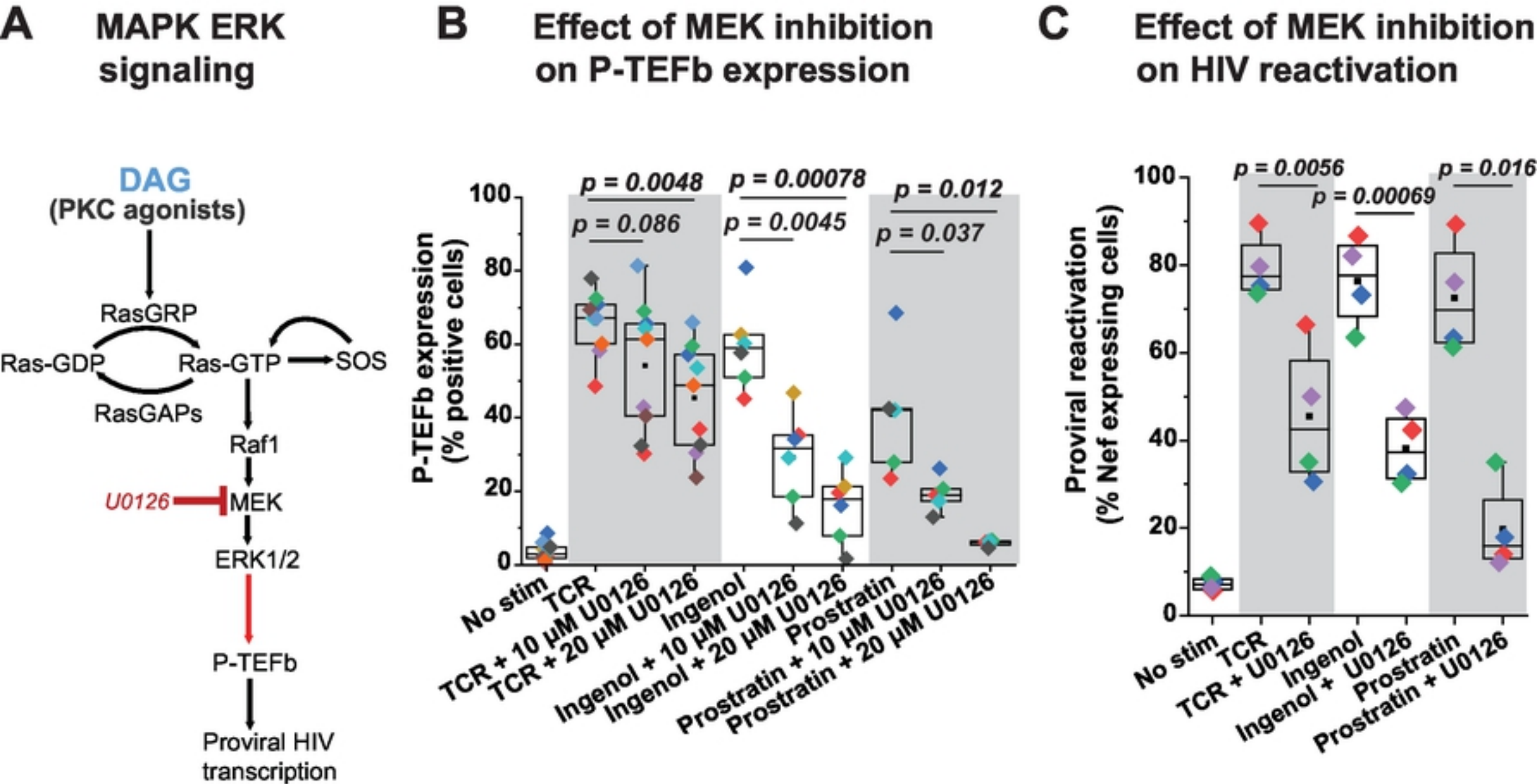
Fig 6

A RasGRP1 membrane trafficking
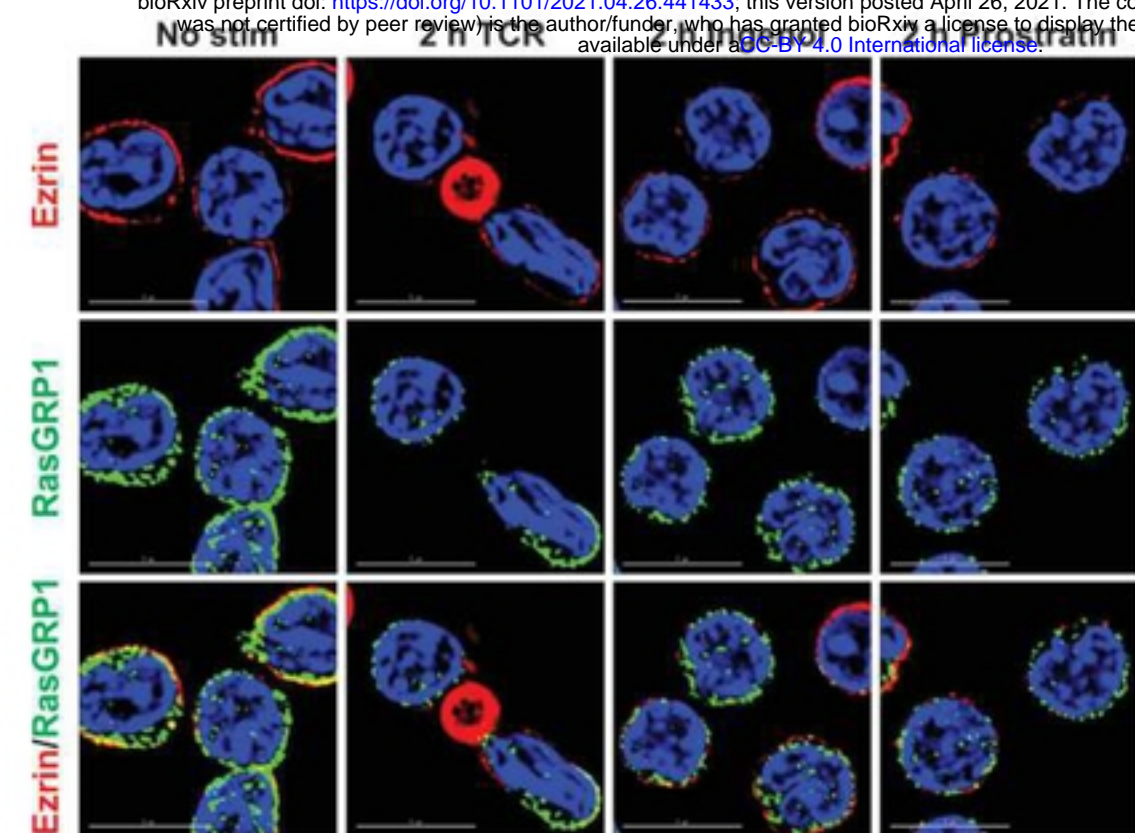
B Extent of RasGRP1 membrane localization
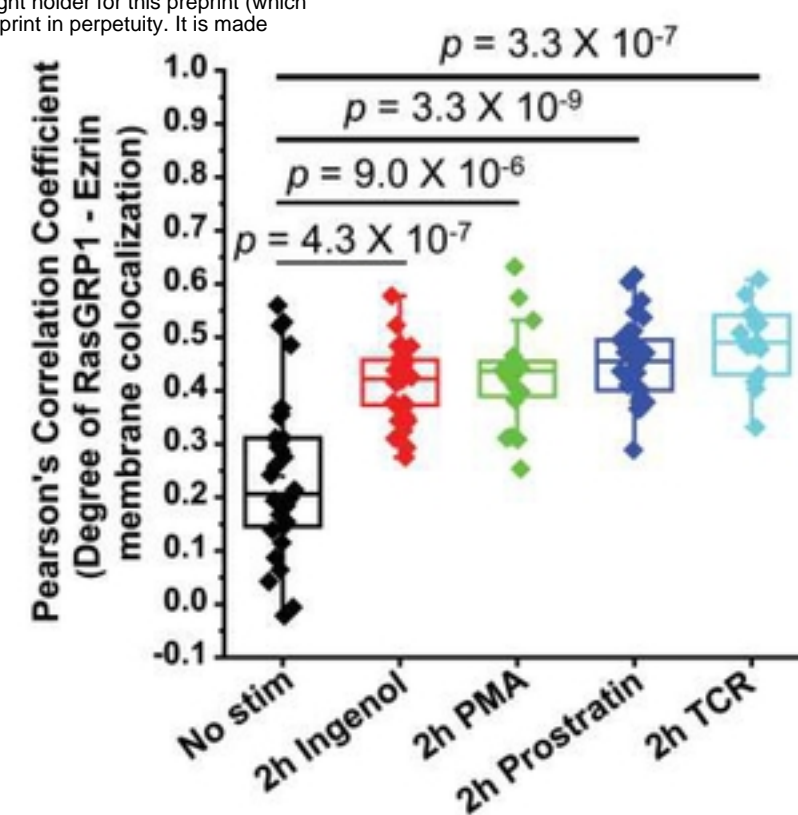
C scRNA profiling of memory T cells
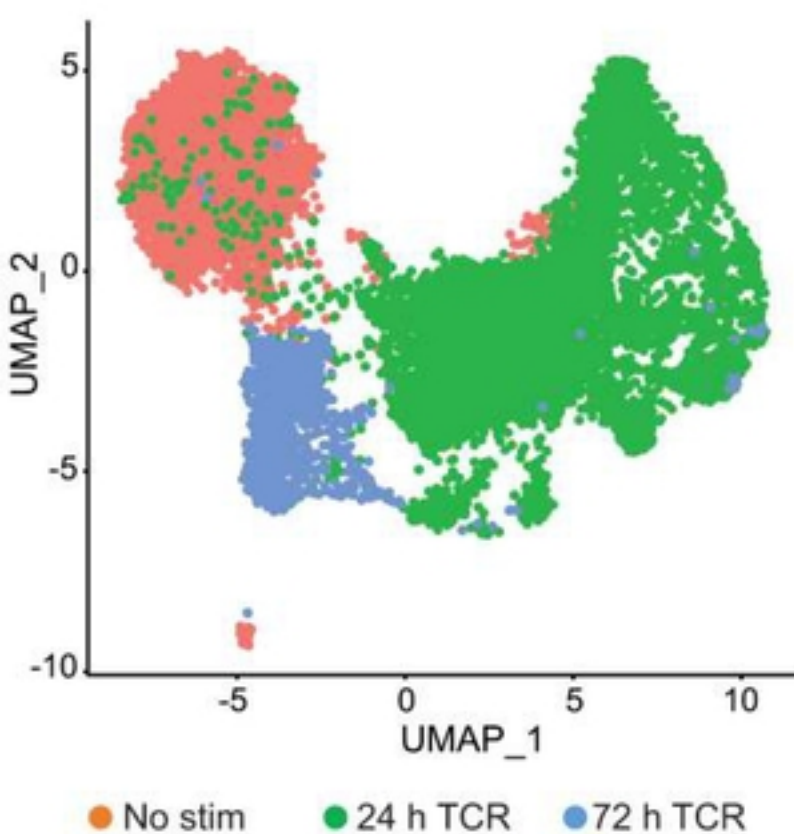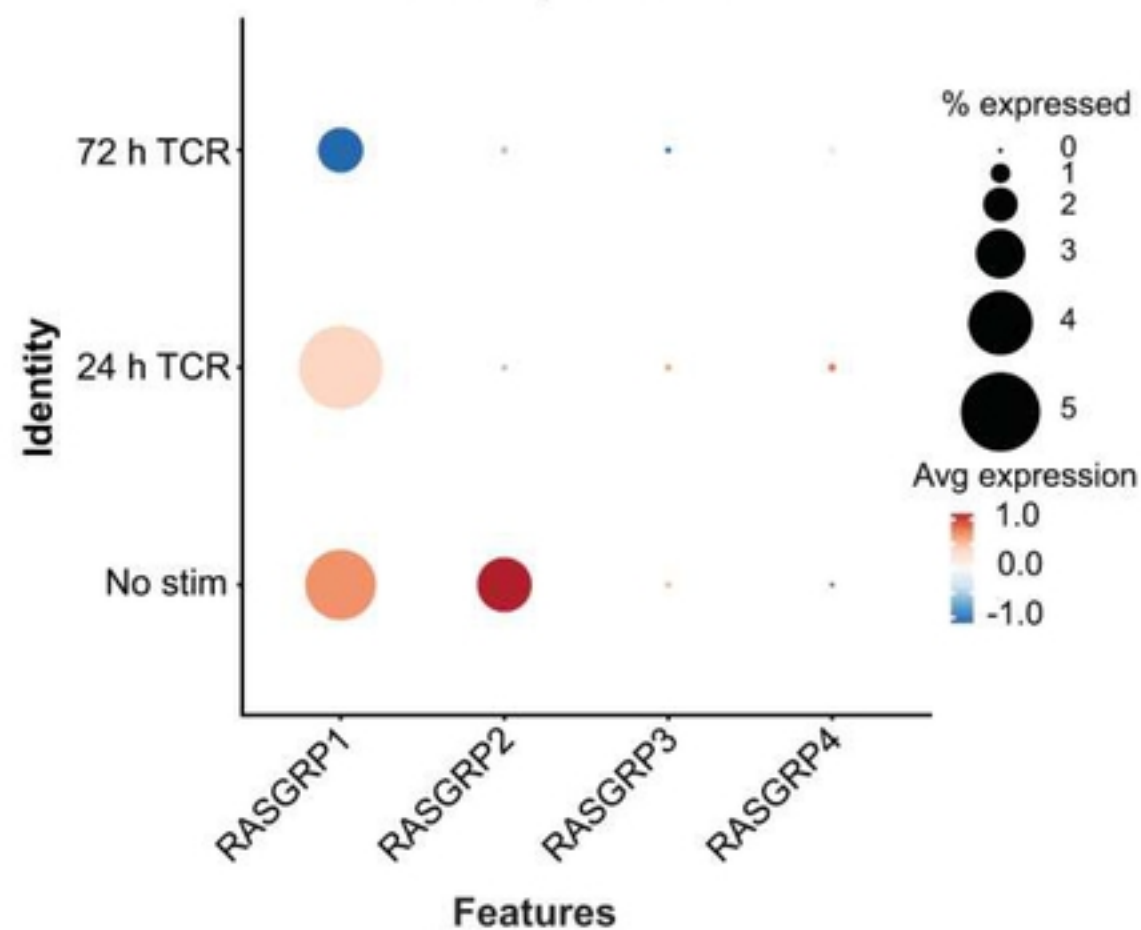
D Expression of RasGRP transcripts in memory T cells

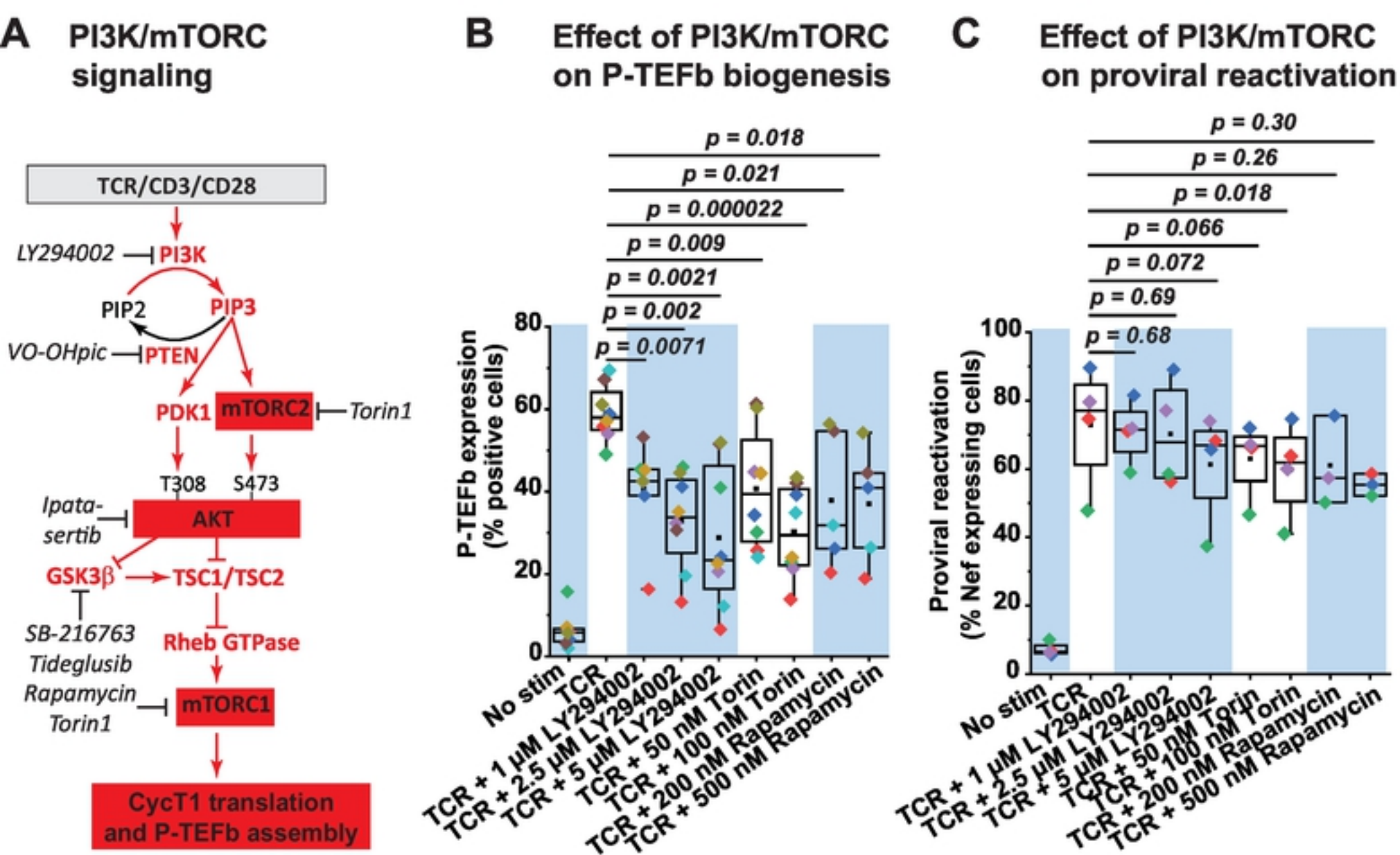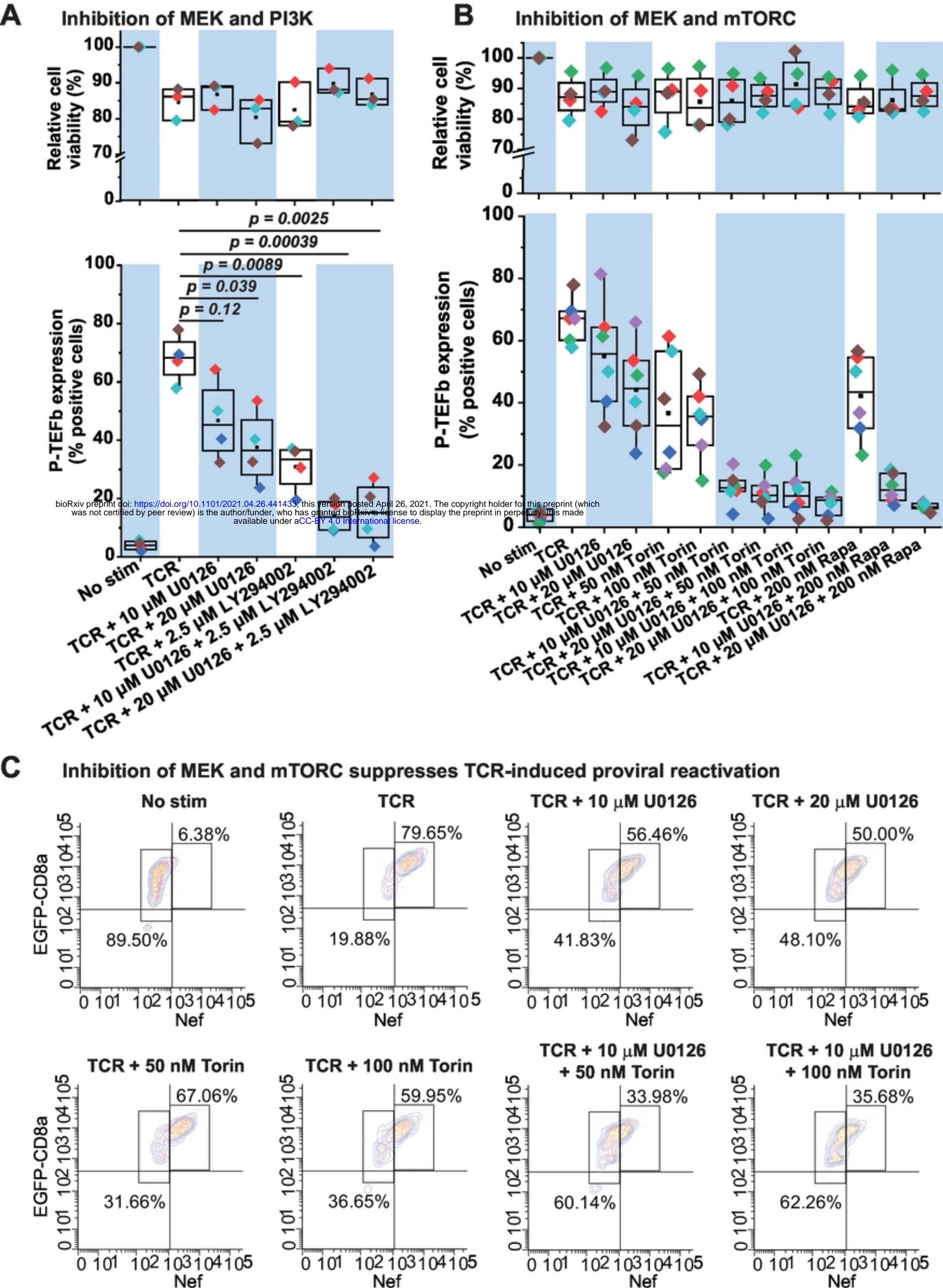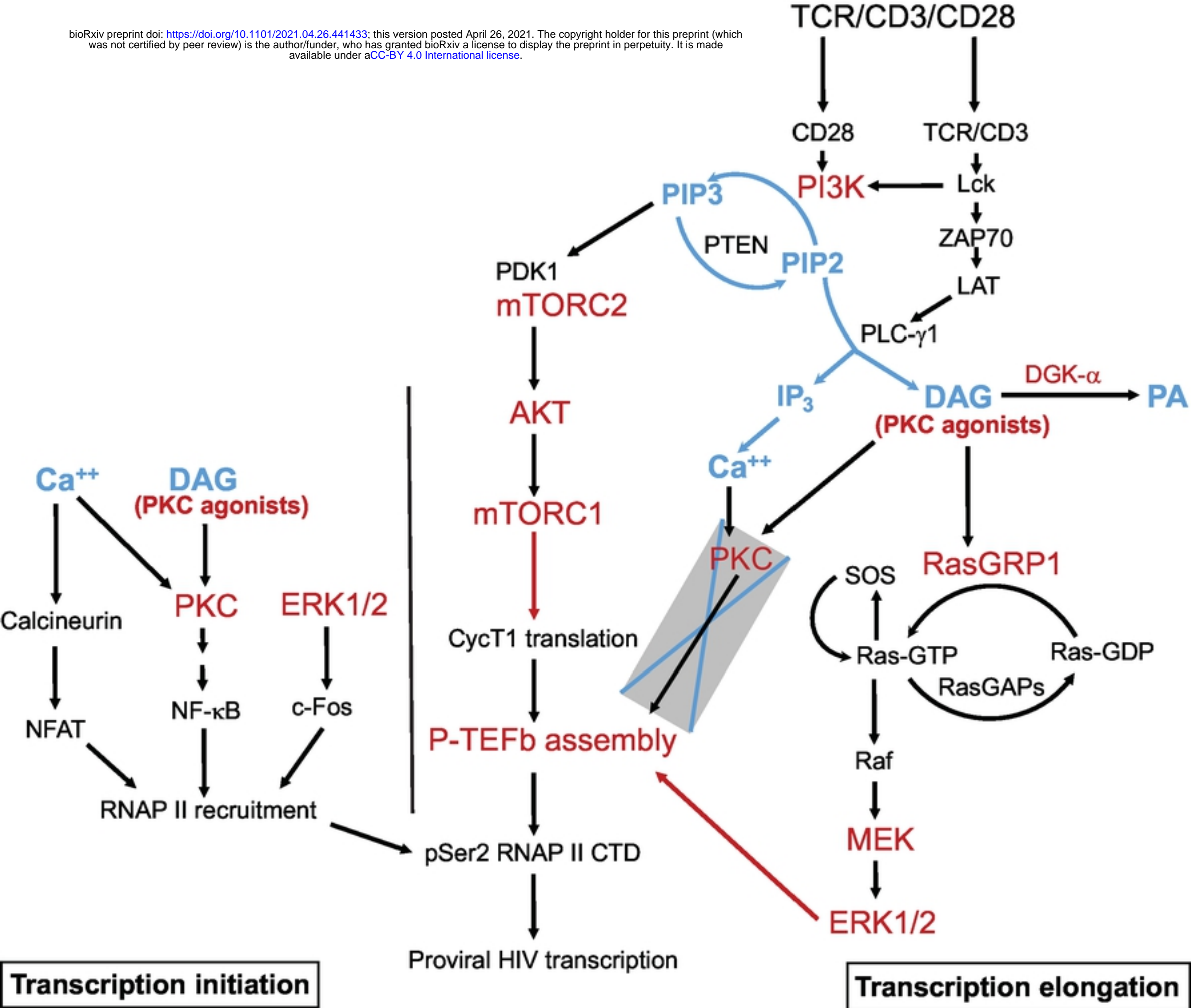Fig 7

A PI3K/mTORC signaling

B Effect of PI3K/mTORC on P-TEFb biogenesis

C Effect of PI3K/mTORC on proviral reactivation

Fig 8

A Inhibition of MEK and PI3K

B Inhibition of MEK and mTORC

C Inhibition of MEK and mTORC suppresses TCR-induced proviral reactivation

Fig 9

Fig 10