A cell-based bioluminescence assay reveals dose-dependent and contextual repression of AP-1-driven gene expression by BACH2

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Abstract

Whereas effector $CD4^{+}$ and $CD8^{+}$ T cells promote immune activation and can drive clearance of infections and cancer, $CD4^{+}$ regulatory T (T_{reg}) cells suppress their function, contributing to both immune homeostasis and cancer immunosuppression. The transcription factor BACH2 functions as a pervasive regulator of immune activation, promoting development of $CD4^{+}$ T_{reg} cells and suppressing the effector functions of multiple effector T cell (T_{eff}) lineages. Here, we report the development of a stable cell-based bioluminescence assay of the transcriptional repressor activity of BACH2. Tetracycline-inducible BACH2 expression resulted in suppression of phorbol 12-myristate 13-acetate (PMA)/ionomycin-driven activation of a luciferase reporter containing BACH2/AP-1 target sequences from the mouse *Ifng* +18k enhancer. BACH2 expression repressed the luciferase signal in a dose-dependent manner but this activity was abolished at high levels of AP-1 signalling, suggesting contextual regulation of AP-1 driven gene expression by BACH2. Finally, using the reporter assay developed, we find that the histone deacetylase 3 (HDAC3)-selective inhibitor, RGFP966, inhibits BACH2-mediated repression of signal-driven luciferase expression. In addition to enabling mechanistic studies, this cell-based reporter will enable identification of small molecule agonists or antagonists of BACH2 function for drug development.

51 **Introduction**

52 CD8$^+$ and CD4$^+$ conventional T (T_{conv}) cells drive immune activation and promote clearance of
53 infections and cancer. However, their function can also provoke autoimmune and allergic
54 inflammation. The immune system therefore employs a variety of suppressive mechanisms,
55 known as immunoregulatory mechanisms, which act both intrinsically within T_{conv} cells and
56 extrinsically to restrain excessive T cell activation. Immunoregulatory mechanisms also suppress
57 beneficial anti-tumour T cell responses to drive deleterious immunosuppression in cancer.
58 Important among extrinsic immunoregulatory mechanisms is the activity of CD4$^+$ regulatory T
59 (T_{reg}) cells which limit T_{conv} cell function and promote immune homeostasis and tumour
60 immunosuppression[1-6]. Immunoregulatory mechanisms are therefore important targets for the
61 development of new therapies aimed at treating inflammatory diseases, disorders of excessive
62 immunopathology and cancer.

63 Appropriate control of T cell differentiation and function requires that they are able to
64 rapidly regulate their gene-expression programs in response to extrinsic signals. Such capacity is
65 provided by transcription factors (TFs), which bind to the available repertoire of regulatory DNA
66 elements in distinct lymphocyte subsets to program cell-type-specific gene expression[7]. Signal-
67 dependent TFs control the response of specific cell types to extrinsic stimuli. In T cells, basic
68 leucine zipper (bZip) TFs of the activator protein 1 (AP-1) family bind to DNA as heterodimers
69 and contribute to activation of gene expression in response to T cell receptor (TCR) signalling[8].
70 AP-1 TFs, including Jun (c-Jun, JunD, JunB), Fos (c-Fos, Fosb, Fosl1, Fosl2) and BATF
71 (BATF1, BATF2, BATF3), contain bZip domains enabling them to form heterodimeric
72 complexes at palindromic 12-O-Tetradecanoylphorbol-13-acetate (TPA) response elements
73 (TRE; 5'-TGA(C/G)TCA-3') within regulatory DNA[9,10]. Upon TCR-signalling, AP-1 complexes

74 translocate to the nucleus where they bind to TRE of genes associated with T_{eff} cell
75 differentiation and function[11].

76 BACH2 is a 92 kDa transcriptional repressor of the bZip TF family and is predominantly
77 expressed in lymphocytes[12]. It functions as an important regulator of immune activation and
78 transcriptional repression. BACH2 intrinsically regulates the differentiation and function of
79 multiple conventional T cell lineages and is required for efficient development of T_{reg} cells.
80 Deficiency of BACH2 results in a cell-intrinsic defect in T_{reg} cell differentiation, such that
81 C57BL/6 syngenic mice lacking BACH2 protein expression develop lethal inflammation[13]. In
82 addition, BACH2 promotes tumour immunosuppression in a T_{reg}-dependent manner[11]. Genetic
83 deletion of *Bach2* in mice results in increased clearance of subcutaneously syngeneic B16
84 melanoma tumours. Furthermore, the *BACH2* gene in humans is a prominent risk locus for
85 multiple autoimmune and allergic diseases[12].

86 The DNA-binding bZip domain of BACH2 is located at the C-terminus of the protein and
87 is required for its repressive activity. In T cells, BACH2 binds to DNA sequences which embed
88 TRE[14]. Through shared possession of bZip domains, BACH2 and AP-1 competitively bind to the
89 same sites within enhancers[11,15]. It has been proposed that such competitive interactions by
90 BACH2 allow it to repress effector-associated gene expression. IFN-γ, encoded by the *Ifng* gene,
91 is an inflammatory cytokine that contributes to type I antiviral and anti-tumour immunity and can
92 contribute to inflammation and immunopathology[16]. *Ifng* expression is markedly elevated in
93 mouse *Bach2*-deficient $CD4^+$ and $CD8^+$ T cells[11,17]. In addition, repression of IFN-γ expression
94 is partially required for BACH2 to promote induced T_{reg} (iT_{reg}) cell induction[13]. These results
95 suggest that repression of IFN-γ expression is a critical biological function of BACH2, but
96 whether these results derive from direct transcriptional repression of the *Ifng* gene has not been

97 formally established. Moreover, the immunoregulatory function of BACH2 and its
98 predominantly lymphocyte-specific gene expression profile make it a potential target in
99 development of therapies for autoimmune diseases and cancer.

100 In this work, we have developed a cell-based assay system to report the transcription
101 factor activity of BACH2, wherein tetracycline-inducible BACH2 expression represses AP-1-
102 driven luciferase activity. Tetracycline-inducible BACH2 expression resulted in suppression of
103 phorbol 12-myristate 13-acetate (PMA)/ionomycin-driven activation of a luciferase reporter
104 containing BACH2/AP-1 target sequences from the mouse *Ifng* +18k enhancer. BACH2
105 expression repressed the luciferase signal in a dose-dependent manner but this activity was
106 abolished at high levels of AP-1 signalling, suggesting contextual control of AP-1 driven gene
107 expression by BACH2. In addition to enabling mechanistic studies, we propose that this cell-
108 based reporter will enable identification of small molecule agonists or antagonists of BACH2
109 function for drug development.

110

111 Results

112 *Generation of a cell line-based luciferase reporter assay of BACH2 repressor function*

113 A putative enhancer of the mouse *Ifng* gene (*Ifng* +18k), containing a canonical TRE and bound
114 by p300, BACH2, and the AP-1 factor JunD in $CD4^+$ and $CD8^+$ T cells was identified (**Fig.
115 1a**)[11]. A short concatenated DNA sequence surrounding the TRE at *Ifng* +18k was subcloned
116 upstream of a minimal promoter (minP) and a luciferase-encoding cDNA sequence (**Fig. 1b**).
117 We additionally subcloned a human BACH2 cDNA inducible expression vector containing a
118 CMV promoter and control elements from the bacterial tetracycline (Tet) resistance operon. We
119 verified the insert and surrounding vector sequences in both constructed plasmids using Sanger

120 sequencing (**Supplementary Fig. 1 and 2**). The luciferase reporter and inducible BACH2
121 expression vectors were co-transfected into Jurkat cells constitutively expressing the Tet
122 repressor protein. Transfected cells were selected using antibiotic selection. Stably transfected
123 single-cell clones were isolated using limiting dilution. A tetracycline-inducible BACH2
124 functional reporter assay was established in addition to a control reporter lacking inducible
125 BACH2 expression (**Fig. 1c**). In the developed system, the Tet repressor binds to a specific
126 sequence upstream of *BACH2* cDNA, inhibiting BACH2 protein expression. The addition of
127 tetracycline results in a conformational change of the Tet repressor protein, preventing its
128 binding, and allowing *BACH2* to be expressed (**Fig. 2a**).

129 We first examined whether BACH2 protein expression is inducible using this system.
130 Cells were treated with or without 1 μg/ml tetracycline, to induce BACH2 expression. Lysates
131 were resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
132 and proteins were detected by western blotting. We observed inducible expression of BACH2
133 protein upon tetracycline treatment of the inducible-BACH2 reporter line but not of the control
134 reporter line, whereas expression of the AP-1 factors JunB, c-Jun and JunD in total cellular
135 lysates was unchanged (**Fig. 2b**).

136 The specificity of anti-BACH2 antibody reactivity was tested by adding a blocking
137 peptide during antibody staining of membranes, which resulted in abolishment of the signal
138 (**Supplementary Fig. 3**). The inducible-BACH2 reporter line treated with and without
139 tetracycline was stimulated with PMA/ionomycin to cause *Ifng* +18k enhancer-driven luciferase
140 expression. A ~40% reduction of luciferase signal was observed in tetracycline-treated cells,
141 which was not observed in the control reporter line (**Fig. 2c**). These results indicate that

142 tetracycline-inducible BACH2 protein represses signal-driven luciferase gene expression
143 controlled by a sequence from the consensus *Ifng* +18k enhancer.
144
145 *Dose-dependent repression of AP-1-driven gene expression by BACH2*
146 To examine the dose-dependency of BACH2-mediated AP-1-driven signal repression, we
147 performed tetracycline titration experiments. Inducible-BACH2 cells were treated with titrated
148 doses of tetracycline and their luciferase activity was determined following PMA/ionomycin
149 stimulation (**Fig. 3a and Supplementary Fig. 4**). BACH2 protein expression was also examined
150 by SDS-PAGE and western blotting (**Fig. 3b**). Luciferase activity was negatively correlated with
151 tetracycline concentration (**Supplementary Fig. 5**) and a significant positive linear correlation
152 between repression of signal-driven luciferase induction and BACH2 protein expression was
153 observed (**Fig. 3c**). Imaging of inducible-BACH2 reporter cells after 6 hours of PMA/ionomycin
154 stimulation also revealed dose-dependent repression of signal-driven luminescence following
155 treatment of cells with tetracycline (**Fig. 4a-b**). These data suggest that BACH2 functions as a
156 dose-dependent repressor of AP-1-driven gene expression regulated by sequences derived from
157 the +18k enhancer of *Ifng*.
158
159 *Contextual dose-dependency of AP-1-driven signal repression by BACH2*
160 BACH2 restrains TCR-driven effector differentiation programmes within $CD8^+$ T cells[11].
161 However, despite possessing high levels of BACH2 expression, naïve T cells are able to
162 differentiate into effector cells in the presence of strong levels of TCR stimulation. We asked
163 whether BACH2-mediated repression of AP-1-driven gene expression occurs to the same extent
164 at any level of AP-1 activation or whether its repressor function is limited at saturating levels of

165 AP-1 activation. We therefore stimulated cells with titrated doses of PMA/ionomycin using a
166 single concentration of tetracycline per titration. Importantly, we observed a loss of BACH2-
167 mediated luciferase signal repression at higher levels of PMA/ionomycin stimulation (**Fig. 5a-b**).
168 These results suggest that BACH2 capacity to mediate AP-1-driven gene expression repression is
169 reduced in the presence of strong activating signals. Thus, dose-dependent AP-1 signal
170 repression by BACH2 is contextual and regulated by the strength of activation signalling in the
171 system.

172

173 *BACH2-mediated repression of signal-driven luciferase induction is inhibited by the HDAC3*
174 *inhibitor RGFP966*

175 There are no known direct activators or inhibitors of BACH2 function. However, it was recently
176 shown that BACH2-mediated repression of the *Prdm1* gene in B cells is partially dependent
177 upon co-recruitment of a complex containing histone deacetylase 3 enzyme (HDAC3). Thus, its
178 repressor function at this locus is inhibited by the HDAC3-selective inhibitor RGFP966[18,19]. We
179 therefore examined whether pre-treatment of cells with RGFP966 would result in inhibition of
180 BACH2-mediated repression in the developed reporter assay. We observed near-complete loss of
181 BACH2-repression of PMA/ionomycin-driven luciferase expression when cells were pre-treated
182 with 12.5 μM RGFP966 (**Fig. 6a-b**). Importantly, RGFP966 treatment did not affect BACH2
183 expression in the assay (**Fig. 6c**). These results provide a positive control for pharmacological
184 inhibition of BACH2 activity in the reporter system developed and shed light on potential
185 mechanisms by which BACH2 represses *Ifng* expression in T cells.

186

187 **Discussion**

188 In this study, we have generated a cell-based luciferase reporter assay system to facilitate
189 analysis of the transcriptional repressor function of BACH2 *in vitro*. Sequences derived from the
190 mouse *Ifng* +18k enhancer sequence bound by both BACH2 and Jun family AP-1 factors in T
191 cells were used to drive luciferase gene expression. Its signal-driven induction was repressed by
192 tetracycline-inducible BACH2 expression[11]. Our inducible BACH2 reporter system suggests that
193 BACH2-mediated repression of AP-1-driven gene expression is dose-dependent and limited at
194 the highest levels of AP-1 signalling. Previous work has shown that BACH2 represses IFN-γ
195 expression, but whether this was the result of direct control of regulatory elements of the *Ifng*
196 gene had not been tested. These findings suggest that BACH2 represses AP-1-driven induction
197 of *Ifng* through regulatory interactions with AP-1 factors at the *Ifng* +18k enhancer
198 (**Supplementary Fig. 6**).

199 In this study, we examined BACH2-mediated regulation of *Ifng* regulatory elements in a
200 Foxp3-negative conventional T cell line. Repression of IFN-γ expression is a critical biological
201 function of BACH2 not only in conventional $CD4^+$ Th1 cells and $CD8^+$ T cells, but also during
202 early iT_{reg} cell development, where BACH2-mediated repression of IFN-γ is required for
203 stabilization of iT_{reg} differentiation prior to Foxp3 induction[11-13]. Moreover, we and others have
204 shown that within lineage-committed $Foxp3^+$ T_{reg} cells, BACH2 is re-purposed and is not
205 required to maintain Foxp3 expression or suppress IFN-γ expression, but rather blocks the TCR-
206 driven transition between resting T_{reg} (rT_{reg}) and activated T_{reg} (aT_{reg}) states[20,21]. Given these
207 observations, we chose to study the regulation of *Ifng* expression by BACH2 in the Foxp3-
208 negative Jurkat cell line. It would be useful in future studies, however, to examine the effect of
209 BACH2 on gene regulation at other loci in a T_{reg} cell line, such as the MT-2 human T_{reg} cell
210 line[22].

211 Stable transfection of the reporter system allowed for the effect of BACH2 on a
212 chromatinized reporter to be determined, as opposed to commonly utilized transfected plasmid
213 luciferase reporters which are not integrated into the host genome and therefore exist as non-
214 chromatinized plasmid DNA. This system provided the opportunity to examine whether
215 BACH2-mediated repression of gene expression in T cells is in part dependent upon regulation
216 of chromatin. Histone deacetylase 3 enzyme (HDAC3) is found in specific complexes containing
217 NCoR1 and NCoR2 and can be recruited to chromatin by transcriptional repressors[23,24]. In B
218 cells, BACH2 has been shown to interact with NCoR1 and NCoR2 resulting in recruitment of
219 HDAC3 to the *Prdm1* gene. As a result, repression of *Prdm1* by BACH2 is dependent upon the
220 activity of HDAC3[18]. Consistent with these findings, we observed that repression of signal-
221 driven luciferase expression by BACH2 was inhibited upon pre-treatment of cells with the
222 HDAC3-specific inhibitor RGFP966. These findings provide an important positive control for
223 inhibition of BACH2-mediated repressor activity in the developed assay system, relevant to
224 development and validation of high-throughput screening assays. It will also be important in
225 future studies to test the extent to which BACH2-mediated repression of *Ifng* expression in
226 primary cells requires the histone deacetylase function of HDAC3.
227 A functional relationship between BACH2 and AP-1 factors underlies T cell memory
228 formation[11,12]. BACH2 inhibits both effector and terminal effector differentiation programmes
229 under conditions of weak TCR-signalling, contributing to differentiation of memory $CD8^+$ T
230 cells and long-lived responses following viral infection[11]. In our assays, loss of BACH2-
231 mediated repression at high levels of stimulation suggests that BACH2-mediated repression is
232 itself regulated by the strength of activating signals that cells receive. This is consistent with a
233 requirement for T cells expressing high levels of BACH2 to nevertheless be able to differentiate

into effector cells in the presence of strong TCR and inflammatory signalling. Indeed, a number of regulatory pathways are known to affect the post-translational stability, localisation and function of BACH2 and an opportunity to further interrogate their role in a reductionist system is provided by this assay. However, such investigations would need to be complemented by corresponding assays using more physiological systems, including in primary T cells.

Finally, this cell-based luciferase reporter system of the transcription factor activity of BACH2 provides an opportunity for identification of small molecule agonists or antagonists of BACH2 function, accomplished through a high-throughput screening approach. Indeed, further work will be required to miniaturize and optimize the assay for use in high-throughput screening. Such assays may enable identification of novel potential therapeutic compounds to either augment or inhibit the suppressive function of BACH2 in immune activation.

Methods

Plasmids and generation of inducible-BACH2 and control reporter cell-based lines

A DNA sequence located at the putative *Ifng* +18k enhancer containing a TRE element (5'–CAAAGAGGATGCCCCG**TGAGTCA**CTTACAAACCACAGC–3') was concatenated three times and subcloned into the hygromycin-resistant luciferase reporter vector pNL2.2 (N107, Promega) upstream of a minimal promoter sequence (minP) and a cDNA sequence encoding luciferase. A human *BACH2* cDNA sequence was sub-cloned into the multiple cloning site (MCS) of the zeocin-resistant tetracycline inducible vector pcDNA.4/TO (V102020, Invitrogen) to generate the pcDNA4/BACH2-inducible vector. Together with pNL2.2 luciferase reporter, both plasmids were co-transfected into blasticidin-resistant Jurkat TRex cells (pcDNA6/TR) using the Amaxa Cell Line Nucleofector Kit V (VCA-1003, Lonza) following the

257 manufacturer's instructions. Stably transfected cells were selected by culturing cells in the
258 presence of 100 μg/ml hygromycin (10687010, Invitrogen) and 200 μg/ml zeocin (R25001,
259 Invitrogen) for two weeks. For the control line, only the hygromycin resistant-pNL2.2 luciferase
260 reporter was transfected and cells were treated with hygromycin alone. Single cell clones were
261 established by limiting dilution. Cells were cultured in RPMI medium (11875085, Gibco)
262 containing 10% tetracycline free fetal bovine serum (P30-3602, PAN Biotech), 50,000 Units of
263 penicillin-streptomycin (15140122, Gibco), 0.1 X glutamax (35050061, Gibco), 0.25 μg/ml of
264 amphotericin B (15290026, Gibco) and 10 μg/ml of blasticidin (R21001, Invitrogen) and
265 maintained after selection in half the concentration of the indicated selection antibiotics at 37 ^{o}C
266 with 5% CO_2.
267

268 *Sanger sequencing and data analysis*

269 Inserts regions of the constructed vectors pNL2.2 *Ifng* +18k reporter and pcDNA.4/BACH2-
270 inducible were confirmed using Sanger sequencing. Primers were designed for sequencing of
271 pNL2.2 *Ifng* +18k reporter vector as follows: Fw: '5–TCGATAGTACTAACATACGC–3' and
272 Rv: '5–GTTGTAGCCGGCTGTCTGTCG–3'. A primer walk strategy was followed to verify the
273 pcDNA.4/BACH2-inducible vector insert and involved designing five different forward and
274 reverse primer sequences as follows: Fw1: '5–CGCAAATGGGCGGTAGGCGTG–3'; Fw2: '5–
275 ACGATGGATTCAGAGACGGC–3'; Fw3: '5–CTTAAGGTCTCTGTTCAGC–3'; Fw4: '5–
276 AATCAAAGTCTGCCCTCG–3'; Fw5: '5–AATTTAGAATGTGAAATCCG–3'; and Rv1: '5–
277 TAGAAGGCACATCGAGG–3'; Rv2: '5–TTTCTCACACACCAATTTGC–3'; Rv3: '5–
278 GAATAGGAAGAGCAGGAGC–3'; Rv4: '5–TCCACACTTTTCGTTATGC–3'; Rv5: '5–
279 TCATCCTCCTCCTCTCCTGC–3'. Sequencing data were analysed using FinchTv 1.4.0

280 software (Geospiza) and ChromasPro 2.1.9 software (Technelysium) for pNL2.2 *Ifng* +18k
281 reporter and pcDNA4/BACH2 inducible-vector respectively. Images of the confirmed insert
282 sequences were merged after data analysis with Adobe Photoshop CS6 software (Adobe Creative
283 Suite 6 Master Collection).
284
285 *Luciferase assay*
286 Clonally derived cell lines were treated with or without tetracycline (T8032, Sigma-Aldrich) for
287 18 hours. Subsequently, cells were stimulated with phorbol 12-myristate 13-acetate (PMA)
288 (P1585, Sigma-Aldrich) and ionomycin (I0634, Sigma-Aldrich) at 25 ng/ml and 1.25 µg/ml
289 respectively, if not otherwise stated, for 6 hours in replenished culture medium containing
290 tetracycline. Luciferase expression was acquired using the Nano-Glo Luciferase Assay System
291 kit (N1130, Promega) following the manufacturer's instructions. Luciferase signal was measured
292 using a PHERAstar FS spectrophotometer. Data were analysed using GraphPad Prism 8
293 software.
294
295 *Western blotting*
296 Selected clones were treated with or without tetracycline for 18 hours. The cells were harvested
297 and washed twice in phosphate-buffered saline (PBS). Cells were lysed in RIPA buffer (89901,
298 Thermo Scientific) containing protease inhibitors (11836170001, Sigma-Aldrich). Total protein
299 concentration was quantified using BCA assay (23225, Thermo Scientific) and normalised
300 protein amount was loaded on SDS-PAGE gels followed by semi-dry western blotting. BACH2
301 protein was detected using BACH2-specific antibody (D3T3G Rabbit mAb, 80775S, Cell
302 Signalling Technology). Detection of Jun family members was conducted with primary anti-c-

303 Jun antibody (N, clone sc-45, J1713, Santa Cruz Biotechnology), anti-JunB antibody (210, clone
304 sc-73, J1813, Santa Cruz Biotechnology) and anti-JunD antibody (329, clone sc-74, A3113,
305 Santa Cruz Biotechnology). As a loading control β-actin protein was stained using anti-β-actin
306 antibody (clone AC-74, A5316, Sigma-Aldrich). The specificity of anti-BACH2 antibody
307 reactivity was tested by adding a BACH2 specific blocking peptide (38475S, Cell Signalling
308 Technology) during primary antibody staining. Stripping of primary and secondary antibodies
309 was performed by incubating the membrane in Restore Western Blot Stripping Buffer (21059,
310 Thermo Scientific) followed by re-probing as described above. Protein quantification was
311 conducted using ImageJ software[25].
312
313 *RGFP966 inhibitor treatment*
314 Inducible-BACH2 reporter cells were plated and pre-treated with or without 1 μg/ml tetracycline
315 for 5 hours prior to RGFP966 (16917, Cayman Chemical Company) inhibitor addition. The
316 tetracycline pre-treated cells were additionally treated with 12.5 μM or without RGFP966 for 12
317 hours following protein extraction or PMA/ionomycin stimulation for 6 hours as described
318 previously. Subsequently, BACH2 protein level detection with western blotting or luciferase
319 activity measurements were performed using methods outlined above.
320
321 *Imaging*
322 Cells from the inducible-BACH2 reporter line were pre-treated with titrated concentrations of
323 tetracycline (namely 0.0024 μg/ml, 0.012 μg/ml, 0.5 μg/ml and 1 μg/ml) or without for 18 hours.
324 Stimulation of cells with PMA/ionomycin at above concentrations followed for 5 hours. Cells
325 were imaged prior and subsequently to luciferase substrate (Nano-Glo Live Cell Assay System

kit (N2011, Promega) addition following the manufacturer's instructions. Luminescence and brightfield images were captured using a Nikon Ti-E microscope, Andor iXon Ultra EM-CCD camera, Nikon 20x 0.8 NA objective, OKO lab environment chamber at 36oC with 5% CO2 and Nikon Elements with JOBS module software. A 3x3 montage of images was acquired in each well with the camera set to maximum sensitivity (300 EM gain, 5.1 amplifier gain) using 10 seconds and 50 ms exposure times for luminescence and brightfield channels respectively. Images were processed and quantified with FIJI[26] using the PureDenoise plug-in[27] to improve the background of the luminescence images and the StarDist plug-in[28] to create cell segmentation masks.

Statistical analysis

Statistical tests of luciferase assays were performed using unpaired two-tailed Student's *t* tests and two-way ANOVA with Bonferroni multiple comparisons correction where specified. All the luciferase measurements were conducted with at least three technical replicates per condition.

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Author contributions

P.V. wrote the manuscript and P.V., T.L. and R.R. designed experiments. P.V., T.L. and S.W. performed experiments. P.V. and H.O. analysed the data. C.B. and J.E. provided reagents and associated protocols. C.B., J.E., S.W. and J.Y. advised on methodology. C.J.I., S.K.W., P.K. and R.R. edited the manuscript. R.R., S.F. and K.O. provided support and/or supervised the work.

Competing financial interests

The authors declare no competing financial interests.

433 **Figure legends**

434 **Figure 1. Design and generation of an inducible cell-based luciferase reporter assay for**
435 **BACH2-mediated repression of AP-1-driven gene expression. a,** Analysis of known BACH2,
436 JunD and p300 binding at the mouse *Ifng* locus as determined by ChIP-Sequencing of $CD8^+$ and
437 $CD4^+$ T cells. A BACH2-bound putative enhancer of *Ifng*, *Ifng* +18k, is indicated by the black
438 triangle. **b,** DNA sequence at *Ifng* +18k containing a TPA response element (TRE; red letters).
439 This sequence was concatenated three times and subcloned upstream of a minimal promoter
440 sequence (minP, grey box) controlling expression of *NlucP* luciferase cDNA sequence in the
441 pNL2.2 reporter vector. **c,** Experimental schema for generation of clonally derived inducible-
442 BACH2 reporter and control reporter lines. Jurkat cells stably transduced with pcDNA6/TR
443 vector resulting in expression of the tetracycline repressor protein were co-transfected with
444 luciferase reporter (pNL2.2 *Ifng* +18k) and inducible expression (pcDNA4/BACH2) vectors.
445 Stably transfected cells were selected with hygromycin and zeocin and then subjected to single-
446 cell cloning resulting in the generation of a luciferase reporter line with the potential for
447 inducible BACH2 expression and a control reporter line lacking BACH2-inducibility.

448

449 **Figure 2. BACH2-mediated repression of AP-1 driven luciferase expression using the**
450 **inducible reporter system. a,** Inducible expression system. Tetracycline repressor (TR) protein
451 in its active form (indicated as a circle) binds to the *TetO₂* sequence upstream of *BACH2* cDNA
452 subcloned into the pcDNA4/BACH2 vector inhibiting transcription of *BACH2*. Tetracycline
453 (Tet) addition changes the conformation and inactivates the tetracycline repressor (TR) protein
454 (indicated as a square), which is subsequently not able to bind to $TetO_2$ sequence, allowing
455 *BACH2* transcription to commence. **b,** Western blot for indicated proteins of total lysates isolated

456 from the clonally derived inducible-BACH2 and control reporter cell lines with or without
457 tetracycline treatment. **c,** Luciferase activity in the inducible-BACH2 and control reporter lines
458 after 6 hours PMA/ionomycin stimulation with or without pre-treatment with tetracycline (1
459 μg/ml). Unpaired two-tailed Student's *t* test (**c**). Data are representative of 3 independent
460 experiments with 3 culture replicates per condition. Bars and error represent mean (SD); ns, not
461 significant; *** $P < 0.001$; **** $P < 0.0001$.
462
463 **Figure 3. Dose-dependent repression of AP-1-driven gene expression by BACH2. a,**
464 Luciferase activity of inducible-BACH2 reporter line after 6 hours PMA/ionomycin stimulation
465 with or without pre-treatment with indicated titrated doses of tetracycline. **b,** Western blot
466 analysis of the abundance of BACH2 protein within total protein lysates from cells in (**a**).
467 Quantified and normalised to β-actin levels of BACH2 protein expression are displayed in the
468 bar graph (top). **c,** Positive correlation between BACH2 expression normalised to β-actin, and
469 luciferase signal repression at the indicated in (**a**) tetracycline concentrations. Two-way ANOVA
470 with Bonferroni correction (**a**) and linear regression analysis (**c**). Data are representative of 2
471 independently repeated experiments with 3 culture replicates per condition. Bars and error
472 represent mean (SD); ns, not significant; *** $P < 0.001$; **** $P < 0.0001$.
473
474 **Figure 4. Bioluminescence imaging of BACH2-reporter cells. a,** Imaging of inducible-
475 BACH2 reporter cells 6 hours after PMA/ionomycin stimulation with or without tetracycline (1
476 μg/ml) treatment. Unstimulated cells were included (indicated as Vehicle). Images were captured
477 after luciferase substrate addition using brightfield (left) and luminescence (right) channels. Each
478 panel is a representative 300 μm x 300 μm cropped area from the overview image. **b,** Frequency

479 of positive luminescent cells in stimulated inducible-BACH2 reporter cells following pre-
480 treatment with the indicated doses of tetracycline.
481
482 **Figure 5. Contextual signal-responsive repression of luciferase expression by BACH2. a,**
483 Luciferase activity in inducible-BACH2 reporter line after 6 hours stimulation with the indicated
484 concentrations of PMA/ionomycin, with or without pre-treatment with titrated tetracycline doses.
485 Concentrations of tetracycline are indicated in the figure legend. **b,** Luciferase signal repression
486 at the indicated PMA/ionomycin concentrations with or without tetracycline (1 μg/ml) pre-
487 treatment. (**a-b**) Data are representative of 2 independently repeated experiments with 3 culture
488 replicates per condition. Bars and error represent mean (SD).
489
490 **Figure 6. BACH2-mediated repression of luciferase expression is inhibited by the HDAC3**
491 **inhibitor molecule RGFP966. a,** and **b,** Luciferase activity in inducible-BACH2 reporter line
492 after 6 hours stimulation with PMA/ionomycin and pre-treatment with or without 1 μg/ml
493 tetracycline and with 12.5 μM of RGFP966 or without (indicated as Veh). **c,** Western blot
494 analysis of the abundance of BACH2 within total protein lysates from cells in (**a-b**) treated with
495 or without 12.5 μM RGFP966. Unpaired two-tailed Student's *t* test (**a-b**). **a-b,** Data are
496 representative of 2 independently repeated experiments with 3 culture replicates per condition.
497 Bars and error represent mean (SD); ns, not significant; **** $P < 0.0001$.
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