

## 1 **HIF2 $\alpha$ is a Direct Regulator of Neutrophil Motility**

2 Sundry Sormendi<sup>1</sup>, Mathieu Deygas<sup>2,3</sup>, Anupam Sinha<sup>1</sup>, Anja Krüger<sup>1</sup>, Ioannis Kourtzelis<sup>1</sup>,  
3 Gregoire Le Lay<sup>2,3</sup>, Mathilde Bernard<sup>2,3</sup>, Pablo J. Sáez<sup>2,3</sup>, Michael Gerlach<sup>4</sup>, Kristin Franke<sup>1</sup>,  
4 Ana Meneses<sup>1</sup>, Martin Kräter<sup>5</sup>, Alessandra Palladini<sup>6</sup>, Jochen Guck<sup>5</sup>, Ünal Coskun<sup>6</sup>,  
5 Triantafyllos Chavakis<sup>1</sup>, Pablo Vargas<sup>2,3</sup>, Ben Wielockx<sup>1</sup>

6  
7 <sup>1</sup>Institute of Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden,  
8 01307 Dresden, Germany. <sup>2</sup> Institut Curie, PSL Research University, CNRS, UMR 144, Paris,  
9 France, <sup>3</sup>Institut Pierre-Gilles de Gennes, PSL Research University, Paris, France, <sup>4</sup>Core  
10 Facility Cellular Imaging (CFCI) at the Faculty of Medicine Carl Gustav Carus, Fetscherstraße  
11 74, 01307 Dresden, Germany. <sup>5</sup>Biotechnology Center, Center for Molecular and Cellular  
12 Bioengineering, Technische Universität Dresden, 01307 Dresden, Germany. <sup>6</sup>Paul Langerhans  
13 Institute Dresden of the Helmholtz Zentrum Munich at the University Hospital and Faculty of  
14 Medicine Carl Gustav Carus of TU Dresden, Germany.

15 **Authorship note:** P.V. and B.W. jointly supervised this work.

16  
17 **Correspondence:** Pablo Vargas, Systems Biology of Cell Polarity and Cell Division, Institut  
18 Curie (UMR144) & Institut Pierre Gilles de Gennes (IPGG), 6 rue Jean Calvin, 75005 Paris,  
19 France., e-mail: [Pablo.Vargas@curie.fr](mailto:Pablo.Vargas@curie.fr) , Phone: +33.1.40795895 and Ben Wielockx, Institute  
20 of Clinical Chemistry and Laboratory Medicine, Technical University Dresden, Fetscherstrasse  
21 74, 01307 Dresden, Germany, e-mail: [Ben.Wielockx@tu-dresden.de](mailto:Ben.Wielockx@tu-dresden.de), Phone:  
22 +49.351.45816260

23

24

25

26

27

28

29

30

31

32

33

34 **Abstract**

35 Orchestrated recruitment of neutrophils to inflamed tissue is essential during initiation of  
36 inflammation. Inflamed areas are usually hypoxic, and adaptation to reduced oxygen pressure  
37 is typically mediated by hypoxia pathway proteins. However, it is still unclear how these  
38 factors influence the migration of neutrophils to and at the site of inflammation either during  
39 their transmigration through the blood-endothelial cell barrier, or their motility in the interstitial  
40 space. Here, we reveal that activation of the Hypoxia Inducible Factor-2 (HIF2 $\alpha$ ) due to  
41 deficiency of HIF-prolyl hydroxylase domain protein-2 (PHD2) boosts neutrophil migration  
42 specifically through highly confined microenvironments. *In vivo*, the increased migratory  
43 capacity of PHD2-deficient neutrophils resulted in massive tissue accumulation in models of  
44 acute local inflammation. Using systematic RNAseq analyses and mechanistic approaches, we  
45 identified RhoA, a cytoskeleton organizer, as the central downstream factor that mediates  
46 HIF2 $\alpha$ -dependent neutrophil motility. Thus, we propose that the here identified novel PHD2-  
47 HIF2 $\alpha$ -RhoA axis is vital to the initial stages of inflammation as it promotes neutrophil  
48 movement through highly confined tissue landscapes.

49

## 50 **Introduction**

51 In the innate immune response, neutrophils represent the first line of protection against  
52 infections, extravasating quickly from circulation to inflamed tissues for fast pathogen  
53 elimination. This process necessitates transit from an oxygen-rich circulatory system to the  
54 inflammation site, which is typically hypoxic due to vasculature damage and/or high metabolic  
55 demand of pathogens and host cells.<sup>1</sup> Thus, neutrophil adaptation to low oxygen levels is  
56 crucial during the early phases of the inflammatory response.

57 Under hypoxic conditions, the transcription factors Hypoxia Inducible Factor-1 (HIF1 $\alpha$ ) and  
58 its isoform HIF2 $\alpha$  are key elements that control immune cell metabolism and function,<sup>2-7</sup> and  
59 importantly, HIF activity is controlled by a class of oxygen sensors known as the HIF prolyl-  
60 hydroxylase domain enzymes (PHD1-3) (reviewed in <sup>8,9</sup>). When oxygen levels decrease, PHDs  
61 get inactivated, which results in HIF $\alpha$  stabilization and transcription of relevant target genes.  
62 Interestingly, HIF1 $\alpha$ -deficiency results in subdued inflammation<sup>2,3</sup> while, inversely, PHD  
63 inactivation and/or HIF $\alpha$  stabilization leads to enhanced neutrophil survival,<sup>4,10</sup> chemotaxis and  
64 degranulation (reviewed in<sup>11</sup>). Although both HIF $\alpha$  subunits have overlapping activities,  
65 unique roles for HIF2 $\alpha$ , including in neutrophil function, have been reported.<sup>4-6</sup>

66 Over the past decade, several mechanisms have been shown to participate in the multi-step  
67 recruitment of neutrophils from circulation to sites of infection or inflammation.<sup>12-14</sup> The  
68 recruitment process requires cell plasticity because cells deform as they move through the  
69 blood-endothelial cell barrier and the confined areas of interstitial tissues. Leucocyte migration  
70 through these microenvironments is orchestrated by actin polymerization regulators, such as  
71 the rho GTPases RhoA, Cdc42 and Rac1.<sup>15-18</sup> In this context, HIF1 $\alpha$  expression has been  
72 suggested to modulate both functional changes in the cytoskeleton and metabolic reprogramming  
73 <sup>19-22</sup>. Importantly, disruption of mechanisms that control neutrophil infiltration in tissues is

74 associated with sepsis, a life-threatening condition with multi-organ failure and one of the  
75 leading causes of death in the intensive care unit (ICU).<sup>23</sup> Conversely, till date, no effective  
76 therapeutic strategies are available for mitigating an uncontrolled neutrophilic inflammatory  
77 response.

78 In this study, we address the effects of PHD2-deficiency on the motility of neutrophils,  
79 including their recruitment during localized inflammation. Using *ex vivo* and *in vivo* imaging  
80 in a variety of highly confined microenvironments, we demonstrate for the first time that HIF2 $\alpha$   
81 over-activation enhances the migratory capacity of neutrophils in a chemotaxis-independent  
82 manner. Through whole transcriptome analysis and combined migratory regulation, we  
83 describe a role for the PHD2-HIF2 $\alpha$ -RhoA axis in the prompt initiation of the innate immune  
84 response.

85

## 86 **Materials and methods**

### 87 **Mice**

88 All mouse strains were housed in our local mouse facility under specific pathogen-free  
89 conditions. Experiments were performed with male and female mice at the age of 8 to 12 weeks.  
90 Vav:cre-PHD2<sup>ff/f</sup> (cKO P2) and Vav:cre-PHD2/HIF2<sup>ff/ff</sup> (cKO P2H2) mouse lines were created  
91 in our laboratory, using PHD2<sup>ff/f</sup>,<sup>24</sup> Vav:cre<sup>25</sup> (generous gift from Dr. Graf, Spain) and/or  
92 HIF2 $\alpha$ <sup>ff/f</sup>.<sup>26</sup> All offspring were born in normal Mendelian ratios and individual floxed lines have  
93 been previously backcrossed to C57BL/6J for at least 9 times. WT controls in all experiments  
94 were Cre-negative littermates without any chimerism (partial deletion of floxed genes in early  
95 blastomeres).<sup>25</sup> Mice were genotyped using primers described in [supplemental Table 1](#) and  
96 knock-down efficiency confirmed via qRT-PCR on isolated neutrophils ([supplemental Figure](#)  
97 [1A, C](#)) and/or genomic PCR on ear biopsies.<sup>27</sup> KRN TCR transgenic mice were inter-crossed

98 with NOD Shilt/J mice (Charles River, Italy) to generate K/BxN mice as described  
99 previously.<sup>28</sup> A detailed description of the inflammation models can be found as supplemental  
100 data. Breeding of all mouse lines and animal experiments were in accordance with the local  
101 guidelines on animal welfare and were approved by the Landesdirektion Sachsen, Germany.

102

### 103 **Histological analysis**

104 5µm thick cryo-sections from 24-hour PMA-treated ears or 7µm thick cryo-sections of knees  
105 from 5-day K/BxN-treated mice were were incubated for 1 hour at 37 °C with primary  
106 antibodies to detect Gr1<sup>+</sup> neutrophils or cCas3<sup>+</sup> apoptotic cells. Imaging was performed using  
107 an epi-fluorescence microscope with Zeiss EC Plan-Neofluar objectives. Number of Gr1<sup>+</sup> cells  
108 per tissue area and percentage of cCas3<sup>+</sup> in Gr1<sup>+</sup> cells were quantified using *Zen* software  
109 Version 3.1 (see [supplemental Table 2](#) for more information on antibodies).

110

### 111 **Flow cytometry**

112 Immune cell profile of synovial fluid from 5-day K/BxN-treated arthritic knee joints was  
113 assessed via FACS performed on LSRII (Becton Dickinson), and cell numbers were counted  
114 on MACS quant (Miltenyi). After knee isolation, digestion to extract the cellular compartment  
115 of the synovial cavity was performed using collagenase D, Dispase II, and DNase I in DMEM.  
116 Knees were incubated for 30 minutes at 37 °C the supernatant was centrifuged, washed and  
117 single cells stained for specific myeloid cell markers using the following fluorophore-  
118 conjugated antibodies for 30 minutes at +4 °C (see [supplemental Table 2](#) for more information  
119 on antibodies).

120

## 121 **Bone Marrow-Derived neutrophils (BMDN)**

122 BMDNs were obtained by crushing long bones the bones in 5% FCS using a mortar and either  
123 isolated by negative selection using the EasySep Mouse Neutrophil Enrichment Kit (Stemcell  
124 Technologies) or by positive selection using biotinylated antibodies (see supplemental data for  
125 more details).

126

## 127 **1-D and 2-D confined migration in micro-channels**

128 Customized polydimethylsiloxane (PDMS) micro devices containing micro-channel areas and  
129 2D-free areas were used to study cell migration in highly confined environments as described  
130 previously.<sup>29</sup> PDMS micro-chips were coated with fibronectin (10 $\mu$ g/ml), their nuclei pre-  
131 labelled with Hoechst for 30 minutes at 37°C and 10<sup>5</sup> neutrophils (in 5 $\mu$ l) loaded in 3 $\mu$ m-  
132 diameter wells. Migrating neutrophils were imaged by video-microscopy (Leica DMI8).  
133 Images were analysed using *Fiji* software<sup>30</sup> and a customised script was used to create  
134 kymographs for individual migrating cells within the micro-channels. Cell speed was  
135 calculated using *MATLAB* software (The MathWorks, Inc). 2D-confined random migration  
136 was analysed using *Imaris* (Bitplane) cell tracking software. Where indicated and prior to their  
137 loading in the PDMS micro-device, cells were resuspended in media containing 1  $\mu$ M of CCG-  
138 100602 (from Sigma), 1  $\mu$ M of ML141 (from Sigma) or 1  $\mu$ M of the Cell permeant C3  
139 transferase (from Cytoskeleton).

140

## 141 **Cell migration in 3D collagen matrices**

142 Neutrophil migration in a 3D environment was evaluated in customized PDMS chambers filled  
143 with varying concentrations of a collagen matrix (3-5 mg/ml) containing 2x10<sup>6</sup> neutrophils/ml.

144 After polymerization of the collagen matrix, phase imaging was performed using video-  
145 microscopy (DMI8). For the chemotaxis assay, CXCL2 (20ng/ml) was added locally to the  
146 matrix.

147

### 148 **Rho-GTPase activity assays**

149 The activity of Activated RhoA, Rac and Cdc42 was measured in lysates from negatively sorted  
150 neutrophils from all genotypes by performing respective activation G-LISA assays (RhoA,  
151 Rac1,2,3 and Cdc42 G-LISA Activation Assays; Cytoskeleton) on fibronectin-coated plates as  
152 per manufacturer's protocol.

153

### 154 **Statistics**

155 Data and graphs represent mean  $\pm$  SEM of representative experiments. Statistical significance  
156 was calculated using the Mann Whitney U test (unpaired) or the Wilcoxon matched-pairs  
157 signed rank test (paired) using GraphPad Prism (v7.02 or higher); \* $p < 0.05$  was considered  
158 statistically significant.

159

### 160 **Data Sharing Statement**

161 RNAseq data are available at GEO (**GSE151703**).

162 Additional data may be found in a data supplement available with the online version of this  
163 article.

164 For original data, please contact [Pablo.Vargas@curie.fr](mailto:Pablo.Vargas@curie.fr) or [Ben.Wielockx@tu-dresden.de](mailto:Ben.Wielockx@tu-dresden.de)

165

166 **Results**

167 **PHD2-deficient neutrophils display enhanced migration in highly confined environments**  
168 **in a HIF-2 $\alpha$ -dependent manner**

169 Although changes in the hypoxia pathway are involved in multiple stages of the inflammatory  
170 response, details on how the PHD/HIF axis governs neutrophil migration remain elusive. Given  
171 that PHD2 is a central regulator of the hypoxia response, we studied the motility of PHD2-  
172 deficient BMDNs isolated from *vav:cre-PHD2<sup>f/f</sup>* mice (henceforth denoted as cKO P2;  
173 [supplementary Figure 1A](#)). Initially, *1D migration assays* in polydimethylsiloxane (PDMS)  
174 micro-channel devices of different levels of constriction (channel widths of 3, 4 or 5 $\mu$ m) were  
175 used to characterize the migratory capacity of individual neutrophils ([Figure 1A](#)).<sup>17,18,31-34</sup>  
176 Interestingly, cKO P2 neutrophils moved significantly faster than their WT counterparts, but  
177 only in the most confined channels ([Figure 1B and Supplemental Figure 1B](#)). To identify  
178 downstream effectors of this phenotype, we evaluated the contributions of HIF2 $\alpha$ , a PHD2  
179 target and a central factor in inflammation<sup>4,35</sup>, in cKO P2H2 neutrophils compared to their  
180 littermate controls ([Supplemental Figure 1C](#)). Interestingly, there were no differences in speed  
181 at any of the degrees of confinement tested ([Figure 1C](#)). These data strongly suggest that  
182 enhanced HIF2 $\alpha$  activation regulates neutrophil motion in very confined microenvironments.

183

184 We extended our analysis to evaluate neutrophil migration in a *2D confined microenvironment*  
185 (4.5  $\mu$ m height) ([Figure 1D](#)). Similar to the results obtained in the 1D migration assay,  
186 neutrophils from cKO P2 mice showed increased motility compared to their WT counterparts,  
187 as evidenced by longer trajectories of equivalent durations ([Figure 1E](#)), as well as greater speed  
188 ([Figure 1F](#)), along with higher mean square displacement (MSD) values ([Figure 1G](#)). On the  
189 other hand, under identical conditions, cKO P2H2 neutrophils did not show any difference in  
190 speed or MSD compared to their WT counterparts ([Figure 1H and 1I](#)). Interestingly, cell



191 migration in a non-confining 2D chamber (12 $\mu$ m height) showed no difference in speed,  
192 trajectories, or MSD ([Supplemental Figure 1D-F](#)). Thus, these data indicate that the PHD2-  
193 HIF2 $\alpha$  pathway regulates cell migration by facilitating mobility strictly in confined spaces.

194

## 195 **PHD2-deficient neutrophils display enhanced non-directed motility in complex** 196 **environments**

197 We used *3D-collagen matrices* to confirm the role of PHD2 in neutrophil migration in a  
198 microenvironment of fibers and different pore sizes, adequately mimicking the tissue  
199 complexity *in vivo*. Therefore, migration of freshly-isolated BMDNs from cKO P2 mice and  
200 WT littermates was compared in dense 3D collagen gels (4mg/ml) ([Figure 2A](#)) during which  
201 cKO P2 neutrophils showed greater motility, as evidenced by a higher displacement radius  
202 ([Figure 2A](#)). Detailed analysis of their random trajectories showed that cKO P2 neutrophils  
203 displayed greater speed and MSD values compared to WT cells ([Figure 2B, C](#)). Interestingly,  
204 this difference was completely lost in less dense collagen gels (2mg/ml) ([Figure 2B and](#)  
205 [Supplemental Figure 2A](#)).

206

207 As it has been suggested that silencing of PHD2 in neutrophils leads to their enhanced  
208 chemotaxis,<sup>36</sup> we assessed this effect in our complex 3D collagen matrix setup using CXCL2  
209 as a classical neutrophil chemokine. Neutrophil trajectory analysis in dense collagen gels  
210 (4mg/ml) showed that absence of PHD2 did not affect neutrophil chemokine sensing because  
211 their directionality towards CXCL2 remained unaltered ([Figure 2D](#)) and a similar strong  
212 increase in cell speed was found in both PHD2-deficient and WT neutrophils ([Figure 2E](#)).  
213 Thus, these results show that PHD2-deficient neutrophils display an enhanced migratory  
214 capacity in dense 3D collagen gels and that PHD2 loss does not affect CXCL2-induced

215 chemotactic capacity. In other words, the faster migration of cKO P2 neutrophils is independent  
216 of chemotaxis induction and is rather linked to enhanced undirected motility or chemokinesis.  
217  
218 The migratory capacity of several cell types in complex microenvironments is highly  
219 dependent on their capacity to deform when encountering narrow pores.<sup>37,38</sup> Therefore, we  
220 evaluated whether cKO P2 neutrophils can overcome severely constricted spaces of only 1µm  
221 width (Figure 2F).<sup>17,39</sup> Remarkably, PHD2-deficient neutrophils showed an enhanced  
222 preference to pass through these constrictions (Figure 2G) and were also faster compared to  
223 WT neutrophils (Figure 2H). Interestingly, under these conditions, cKO P2H2 neutrophils  
224 showed reduced migration; and, similar migration kinetics than WT cells (Figure 2I, J), again  
225 suggesting a PHD2/HIF2 $\alpha$ -dependent axis in migration through extreme narrow constrictions.  
226  
227 Next, we studied whether the ability of cKO P2 neutrophils to pass through small confinements  
228 is related to changes in their deformability when an external force is applied. For this, we first  
229 analyzed neutrophil deformability using *real time fluorescence and deformability cytometry*  
230 (RT-FDC) (see supplemental data), which can extract the stiffness of cells (Young's Modulus)  
231 in high-throughput, without contact at ms-timescales.<sup>40,41</sup> We used steady-state BMDNs,  
232 Phorbol 12-Myristate 13-Acetate (PMA)-activated BMDNs, and peripheral blood neutrophils  
233 isolated at 6h after thioglycolate-induced peritonitis. However, no differences were observed  
234 between cKO P2 and WT neutrophils under any of the conditions tested (supplemental Figure  
235 2B). Likewise, a *microcapillary microcirculation mimetic* (MMM) assay<sup>42,43</sup> using peritonitis  
236 neutrophils showed no difference in their ability to passively navigate through multiple  
237 constrictions at high speed (supplemental Figure 2C). Taken together, these assays strongly  
238 suggest that loss of PHD2 does not affect neutrophil deformability under externally applied  
239 stress without confinement.

240

241 **PHD2-deficient neutrophils extravasate faster *in vivo* and accumulate in inflamed tissue**

242 Based on the enhanced ability of PHD2-deficient neutrophils to overcome very small  
243 constrictions, we decided to study the behavior of these cells *in vivo*; specifically, in a more  
244 complex setting of sterile skin inflammation. Ear lobes from cKO P2 and WT littermate mice  
245 that displayed no difference in total numbers of hematopoietic stem cells, myeloid progenitors  
246 or mature neutrophils ([supplemental Figure 3A](#)), were ectopically treated with PMA and the  
247 recruitment of Ly6G<sup>+</sup> cells was visualized using intra-vital 2-photon microscopy ([Figure 3A](#)).  
248 In line with our previous experiments, we found that PHD2-deficient neutrophils were able to  
249 extravasate about 30% faster from the vessel into the ear tissue than their WT counterparts  
250 ([Figure 3B-C](#)). Furthermore, the cumulative effect of faster neutrophil extravasation time  
251 resulted in an anticipated increase in Gr1<sup>+</sup> cells in the inflamed cKO P2 ear compared to that  
252 in WT littermates at 24 hours after PMA-treatment ([Figure 3D](#)). Conversely but consistently,  
253 this difference in migration was abolished in cKO P2H2 mice ([Figure 3E](#)), further confirming  
254 a role for HIF2 $\alpha$  activity in driving increased migration capacity of these neutrophils.

255 As previous studies have described PHD2-related improved survival of neutrophils during  
256 inflammation,<sup>4,44</sup> we evaluated the level of apoptotic cells in 24 hour PMA-treated ears, but  
257 found no difference in cleaved caspase-3<sup>+</sup> cell numbers (cCas3<sup>+</sup>) between the different  
258 genotypes ([Figure 3F, G](#); [supplemental Figure 3B, C](#)). Additionally, as recent work has  
259 associated PHD2 with enhanced neutrophil glycolysis and their recruitment to sites of  
260 inflammation,<sup>36</sup> we assessed the glycolytic capacity of BMDNs from cKO P2, cKO P2H2, and  
261 their respective WT counterparts by measuring extracellular acidification rate (ECAR). In line  
262 with previous reports, PHD2-deficient neutrophils appeared to be significantly more glycolytic  
263 than their respective WT counterparts ([Figure 3H](#)). However, neutrophils lacking both PHD2

264 and HIF2 $\alpha$  also showed significantly higher glycolysis (Figure 3I). Taken together, although  
265 HIF2 $\alpha$  directly controls the migration speed of neutrophils in confined spaces and inflamed  
266 tissues, this effect is independent of their survival or glycolytic activity.

267

### 268 **HIF2 $\alpha$ stabilization upon loss of PHD2 affects cytoskeletal gene expression profiles**

269 It is well-accepted that the functionality of innate immune cells varies depending on the lipid-  
270 type composition of its cytoplasmic membrane.<sup>45,46</sup> Therefore, we evaluated if altered  
271 membrane lipid composition of the cKO P2 neutrophils could account for their different  
272 migratory ability, by performing high-throughput lipidomic analysis of freshly isolated  
273 BMDNs (see supplemental data). However, as there were no significant alterations between  
274 the cKO P2 and WT BMDNs (supplemental Figure 2D and Table 3), it appears unlikely that  
275 differences in the lipid composition are directly responsible for the dramatic difference in the  
276 migratory capacity of the cKOP2 neutrophils.

277

278 Next, to further characterize the molecular underpinnings of the HIF2 $\alpha$ -driven neutrophil  
279 migration phenotype, we used *next generation sequencing* (NGS) wherein the steady state  
280 transcriptome of BMDNs derived from cKO P2 and cKO P2H2 mice were analyzed and  
281 compared to that from their respective WT counterparts (Figure 4A). Gene signatures of  
282 various lineages were evaluated using gene set enrichment analyses (GSEA) as described  
283 previously.<sup>47-49</sup> In line with our *in vivo* cCas3+ results, we detected no significant apoptosis  
284 signatures among any of the genotypes (Figure 4B) and NGS confirmed a significant  
285 enrichment of glycolysis/gluconeogenesis related genes in both cKO P2 and cKO P2H2  
286 BMDNs (Figure 4C). Strikingly, steady state BMDNs lacking PHD2, with or without HIF2 $\alpha$ ,  
287 displayed a significant reduction in genes related to the innate immune response but not the

288 chemokine signaling pathway (Figure 4D). Together, these observations suggest that  
289 significant HIF2 $\alpha$ -independent changes in glycolytic capacity and immune response of PHD2-  
290 deficient neutrophils can be likely linked to HIF1 $\alpha$  activity, as previously suggested.<sup>2,36</sup>  
291 Conversely, a number of HIF2 $\alpha$ -dependent gene signatures associated with PHD2 deficiency  
292 related to function and structure of the neutrophil cytoskeleton, including Rho GTPase activity  
293 (Figure 4E). Additionally, using an integrative method, we identified a number of HIF2 $\alpha$ -  
294 associated *master regulators* that could potentially control cellular cytoskeletal rearrangements  
295 through transcriptional or protein regulation (supplemental Figure 4A).

296

### 297 **Diminished RhoA GTPase activity underlies the faster HIF2 $\alpha$ -dependent migration of** 298 **PHD2-deficient neutrophils**

299 Small Rho GTPases (RhoA, cdc42 and Rac) are the final molecular effectors that steer  
300 cytoskeletal dynamics. In line with this, we identified numerous potential associations (direct  
301 and/or indirect) among 49 genes/proteins and with RhoA and/or Cdc42 (bold lines), but not  
302 Rac GTPase (Figure 5A). Notably, 7 of these genes have been previously identified as being  
303 associated with HIF2 $\alpha$  binding sites (supplemental Figure 4B).<sup>50</sup>

304 To substantiate this link between PHD2/HIF2 $\alpha$  and Rho GTPases, we used an *ex vivo*  
305 enzymatic assay to quantify the activity of these Rho GTPases in untreated freshly-isolated  
306 BMDNs from cKO P2 and P2H2 mice. Interestingly, cKO P2 neutrophils exhibited diminished  
307 RhoA and Cdc42 GTPase activity (Figure 5B, C), while Rac GTPase activity was comparable  
308 with WT neutrophils (Figure 5D). Further, cKO P2H2 neutrophils displayed no significant  
309 reduction in either RhoA, Cdc42 or Rac GTPase activity, suggesting that regulation of RhoA  
310 and/or Cdc42 is dependent on the PHD2/HIF2 $\alpha$ -axis (Figure 5B-D).

311 Given this reduction in RhoA and Cdc42 GTPase activity in PHD2-deficient neutrophils, we  
312 examined whether their direct inhibition in WT neutrophils can mimic the motility phenotype

313 displayed by cKO P2 neutrophils. Therefore, we performed a series of *ex vivo* 1D-migration  
314 assays using the RhoA inhibitor CCG100602 (CCG) or the Rho inhibitor Exoenzyme C3  
315 Transferase (C3) and found that while the use of low doses of CCG or C3 enhanced the speed  
316 of migrating neutrophils in 3 $\mu$ m micro-channels (Figure 5E), treatment of cells with a Cdc42  
317 inhibitor (ML141) did not have any effect on the velocity of BMDNs (Figure 5F). Taken  
318 together, our data strongly argue for a PHD2/HIF2 $\alpha$ -orchestrated regulatory loop in RhoA  
319 GTPase activity-dependent motility of BMDNs.

320

### 321 **The PHD2/HIF2 $\alpha$ -axis controls neutrophil accumulation in joints during severe** 322 **inflammatory arthritis**

323 To test the biological effects of the enhanced migratory capacity of PHD2-deficient  
324 neutrophils, we subjected the different mouse strains to an autoantibody-induced inflammatory  
325 arthritis model (K/BxN), which has been shown to be myeloid dependent (Figure 6A).<sup>51,52</sup> cKO  
326 P2 mice displayed enhanced swelling of the hind limbs compared to their WT littermates  
327 (Figure 6B) and this effect was sustained throughout the first 2 weeks of the experiment. In  
328 line with our previous results, cKO P2H2 and their WT littermates displayed no difference in  
329 swelling (Figure 6C). To characterize the myeloid composition of the inflamed knee joints, we  
330 performed flow cytometry analysis of the synovial fluid drawn on day 5, which revealed much  
331 higher accumulation of neutrophils in cKO P2 knee joints (>3-fold increase versus WT), along  
332 with slightly enhanced macrophages (Figure 6D); immunofluorescence for Gr1 on knee joints  
333 further confirmed this observation (Figure 6E). Conversely, although no differences were  
334 observed in joint swelling between cKO P2H2 mice and their WT littermates, their synovial  
335 fluid showed a slight but significant reduction in neutrophil numbers at day 5 compared to cKO  
336 P2 mice (Figure 6F). Thus, also in arthritic joints PHD2/HIF2 $\alpha$  is a central axis during the  
337 initial stages of the inflammation.

338

## 339 **Discussion**

340 In the current work we have explored if hypoxia pathway proteins can directly regulate  
341 neutrophil motility, and reveal that activation of HIF2 $\alpha$  in mouse neutrophils due to constitutive  
342 PHD2 loss enhances neutrophil migration through very confined environments independent of  
343 chemotactic -, glycolytic- or apoptotic-activity. Using a combination of *in vivo*, *ex vivo* and  
344 deep sequencing approaches, we provide evidence that these neutrophils have the capacity to  
345 migrate faster than their WT counterparts, and that this phenotype may be directly related to  
346 changes in their cytoskeleton mediated by a substantial reduction in RhoA GTPase activity.

347 Although it is generally accepted that neutrophils are the first immune cells to arrive in the  
348 tissue during inflammation, the molecular basis of neutrophil recruitment, which encompasses  
349 extravasation and interstitial migration, remain elusive. Further, neutrophil recruitment has  
350 been evaluated using a variety of migration assays in multiple studies related to the innate  
351 immune response,<sup>53-56</sup> including in the context of hypoxia pathway proteins,<sup>2,4</sup> but these studies  
352 call into debate the role of adhesion molecules.<sup>57,58</sup> Here, we consistently show in 1D, 2D and  
353 3D assays that neutrophils lacking PHD2 alone, and not both PHD2 and HIF2 $\alpha$ , display  
354 enhanced cell motility and that only in severely confined environments. This difference in  
355 chemokinesis between cKO P2 and WT remained in a comparable setup using a chemokine as  
356 attractant (chemotaxis), demonstrating that the enhanced migratory capacity regulated by the  
357 PHD2-HIF2 $\alpha$  axis is probably a cell intrinsic characteristic.

358 An important process during neutrophil recruitment is the final and time-limiting step of trans-  
359 endothelial migration (TEM), which is, in part, mediated by mechanical forces generated by  
360 the migrating neutrophil itself.<sup>59-61</sup> We reveal a central role for HIF2 $\alpha$  in this process. Indeed,  
361 considering the narrow pores between neighboring endothelial cells during the early phase of



362 neutrophil diapedesis,<sup>60</sup> our results from multiple approaches reiterate two main observations,  
363 viz., that greater numbers of cKO P2 neutrophils pass through small constrictions with  
364 enhanced speed. Intuitively, these observations account for the shorter TEM-time in a local ear  
365 inflammation model. The cumulative effects of such enhanced transmigration into inflamed  
366 tissues that were observable even at later time points in two completely independent *in vivo*  
367 models, i.e., inflammatory skin lesions and sterile arthritis. Indeed, it is possible that the  
368 enormous increase in cKO P2 neutrophils is positively affected by the fact that once a pore is  
369 opened, successive neutrophils are more likely to extravasate at this spot, enabling more  
370 neutrophils to enter the interstitium (skin) or the synovium (joint) of the inflamed tissue.<sup>60</sup>

371 Previous studies in a model of acute lung injury have reported enhanced glycolytic capacity of  
372 PHD2-deficient neutrophils, potentially due to HIF1 $\alpha$  stabilization, which also enhanced  
373 neutrophil recruitment to the inflammatory site.<sup>36</sup> Here, we confirm enhanced glycolysis in  
374 cKO P2 neutrophils and show that it is HIF2 $\alpha$ -independent, strongly suggesting that glycolytic  
375 metabolism does not underlie the chemokinesis phenotype described here. The absence of  
376 differences in neutrophil apoptosis *in vivo* was corroborated by the RNAseq data from both  
377 single and double knock-out neutrophils, implying that the prolonged inflammation phenotype  
378 in cKO P2 mice was probably not due to persistence of the neutrophils. This is in contrast to  
379 results obtained using *in vitro* approaches that describe reduced apoptosis in HIF2 $\alpha$  over-  
380 expressing neutrophils, which then resulted in delayed resolution of the inflammatory  
381 response.<sup>4</sup> This group also reported delayed apoptosis in PHD2-deficient neutrophils and  
382 connected this to persistent inflammation.<sup>36</sup> We believe these discrepancies are related to  
383 differences in the experimental models used.

384 Although several studies have linked the hypoxia pathway to the migratory capacity of a cell,  
385 only a few have suggested a role for the PHD/HIF axis in regulating cell migration through  
386 changes in cytoskeletal function.<sup>20-22</sup> In migrating neutrophils *in vivo*, dynamic polymerized



387 actin converges at the leading-edge of pseudopods, while stable actin with high acto-myosin  
388 contractility assemble at the rear. Both polarization and maintenance of this cytoskeletal  
389 asymmetry strongly rely on Rho GTPase activity.<sup>62,63</sup> Using deep sequencing data from  
390 neutrophils of single and double transgenic lines, we show that a vast number of genes  
391 associated with Rho GTPase signaling are either directly or indirectly regulated by HIF2 $\alpha$ .  
392 Interestingly, cKO P2 neutrophils displayed a significant downregulation of RhoA GTPase and  
393 we show this to be directly associated with enhanced motility because RhoA-inhibitor treated  
394 WT neutrophils behaved similarly in confined environments. These findings are similar to  
395 those reported earlier, i.e., increased flux of RhoA-deficient neutrophils and aggravated tissue  
396 injury in LPS-induced acute lung injury.<sup>64</sup> A potential explanation is that the partial RhoA  
397 inhibition would primarily decrease dynamic cell protrusions, known to restrict cell migration  
398 by competing with stable actin cables at the cell rear.<sup>18</sup> Alternatively, the HIF2 $\alpha$  axis could be  
399 directly involved in the induction of cell contractility, which promotes neutrophil and DCs  
400 migration under strong confinement.<sup>17,34</sup> However, further efforts are required to identify the  
401 specific molecular mechanism.

402 In conclusion, our results demonstrate that HIF2 $\alpha$ -activation, due to constitutive loss of PHD-  
403 2, enhances the motility of neutrophils in highly confined surroundings, also during  
404 inflammation. Importantly, this phenotype is independent of chemotaxis signaling, glycolysis  
405 or apoptosis. Mechanistically, it is the reduction of RhoA GTPase activity that enhances the  
406 motility of PHD2 deficient neutrophils through very confined microenvironments. These  
407 findings highlight the potential deleterious effects of sustained HIF2 $\alpha$  activity and may have  
408 important implications for the uncontrolled use of hypoxia mimetic agents that are currently  
409 licensed or are in phase II and III clinical trials.

410

411 **Conflict-of-interest**

412 The authors have declared that no conflict of interest exists.

413

414 **Acknowledgments**

415 S.S. received financial support from the Dresden International Graduate School for  
416 Biomedicine and Bioengineering (DIGS-BB), B.W. was supported by the Heisenberg program  
417 (Deutsche Forschungsgemeinschaft – DFG, Germany; WI3291/5-1 and 12-1). This work was  
418 supported by grants from the DFG (TRR-CRC 205 Die Nebenniere: Zentrales Relais in  
419 Gesundheit und Krankheit (A02) to B.W. and T.C.; CRC 1181 (C7) to T.C.; the Alexander von  
420 Humboldt Foundation (AvH Professorship to J.G.). PV received financial support from the  
421 Association Nationale pour la Recherche (MOTILE project, ANR-16-CE13-0009), the  
422 Emergences Canceropole (SYNTEC project) and Labex-IPGG, as well as from "Institut Pierre-  
423 Gilles de Gennes" (laboratoire d'excellence, "Investissements d'avenir" program ANR-10-  
424 IDEX-0001-02 PSL and ANR-10-LABX-31). We would like to thank Silke Tulok and Dr.  
425 Anja Nobst from Core Facility Cellular Imaging (CFCI-MTZ-Dresden) for excellent  
426 assistance, Dr. Graf (CRG, Barcelona, Spain) for the Vav:cre mouse line and Dr. Vasuprada  
427 Iyengar for English Language and content editing.

428

429 **Authorship**

430 S.S. designed the study, performed the majority of experiments, analysed data, and contributed  
431 in writing the manuscript. M.D., I.K., K.F. and M.K. designed and performed experiments,  
432 analysed data and contributed to the discussions. P.J.S., GLL and MB analysed data and  
433 contributed to the discussions. A.S. performed deep sequencing analysis and A.P. performed  
434 lipidomic analysis. M.G. performed intravital microscopy and contributed to the discussions.

435 A.K. and A.M. performed experiments and analysed data. J.G., Ü.C. provided tools and  
436 contributed to the discussions. T.C. provided tools, contributed to the discussions and edited  
437 the manuscript. P.V. designed and supervised the ex vivo migration studies, performed  
438 experiments, analysed data, and contributed in writing the manuscript. B.W. designed and  
439 supervised the overall study, analysed data, and wrote the manuscript.

440

441 **Current affiliation:** I.K.: Hull York Medical School, York Biomedical Research Institute,  
442 University of York, York, United Kingdom. M.K. and J.G.: Max Planck Institute for the  
443 Science of Light & Max-Planck-Zentrum für Physik und Medizin, 91058 Erlangen, Germany.

444 **References**

- 445 1. Taylor CT, Colgan SP. Regulation of immunity and inflammation by hypoxia in  
446 immunological niches. *Nat Rev Immunol*. 2017;17(12):774-785.
- 447 2. Cramer T, Yamanishi Y, Clausen BE, et al. HIF-1 $\alpha$  is essential for myeloid cell-  
448 mediated inflammation. *Cell*. 2003;112(5):645-657.
- 449 3. Peyssonnaud C, Datta V, Cramer T, et al. HIF-1 $\alpha$  expression regulates the  
450 bactericidal capacity of phagocytes. *J Clin Invest*. 2005;115(7):1806-1815.
- 451 4. Thompson AA, Elks PM, Marriott HM, et al. Hypoxia-inducible factor 2 $\alpha$  regulates  
452 key neutrophil functions in humans, mice, and zebrafish. *Blood*. 2014;123(3):366-376.
- 453 5. Dai Z, Li M, Wharton J, Zhu MM, Zhao YY. Prolyl-4 Hydroxylase 2 (PHD2)  
454 Deficiency in Endothelial Cells and Hematopoietic Cells Induces Obliterative Vascular  
455 Remodeling and Severe Pulmonary Arterial Hypertension in Mice and Humans Through  
456 Hypoxia-Inducible Factor-2 $\alpha$ . *Circulation*. 2016;133(24):2447-2458.
- 457 6. Imtiyaz HZ, Williams EP, Hickey MM, et al. Hypoxia-inducible factor 2 $\alpha$  regulates  
458 macrophage function in mouse models of acute and tumor inflammation. *J Clin Invest*.  
459 2010;120(8):2699-2714.
- 460 7. Clever D, Roychoudhuri R, Constantinides MG, et al. Oxygen Sensing by T Cells  
461 Establishes an Immunologically Tolerant Metastatic Niche. *Cell*. 2016;166(5):1117-1131  
462 e1114.
- 463 8. Sormendi S, Wielockx B. Hypoxia Pathway Proteins As Central Mediators of  
464 Metabolism in the Tumor Cells and Their Microenvironment. *Front Immunol*. 2018;9:40.
- 465 9. Watts ER, Walmsley SR. Inflammation and Hypoxia: HIF and PHD Isoform  
466 Selectivity. *Trends Mol Med*. 2019;25(1):33-46.

- 467 10. Walmsley SR, Chilvers ER, Thompson AA, et al. Prolyl hydroxylase 3 (PHD3) is  
468 essential for hypoxic regulation of neutrophilic inflammation in humans and mice. *Journal of*  
469 *Clinical Investigation*. 2011;121(3):1053-1063.
- 470 11. Lodge KM, Cowburn AS, Li W, Condliffe AM. The Impact of Hypoxia on Neutrophil  
471 Degranulation and Consequences for the Host. *Int J Mol Sci*. 2020;21(4).
- 472 12. Hajishengallis G, Chavakis T. Endogenous modulators of inflammatory cell  
473 recruitment. *Trends Immunol*. 2013;34(1):1-6.
- 474 13. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and  
475 inflammation. *Nat Rev Immunol*. 2013;13(3):159-175.
- 476 14. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation:  
477 the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7(9):678-689.
- 478 15. Cernuda-Morollon E, Ridley AJ. Rho GTPases and leukocyte adhesion receptor  
479 expression and function in endothelial cells. *Circ Res*. 2006;98(6):757-767.
- 480 16. Salvermoser M, Pick R, Weckbach LT, et al. Myosin 1f is specifically required for  
481 neutrophil migration in 3D environments during acute inflammation. *Blood*.  
482 2018;131(17):1887-1898.
- 483 17. Thiam HR, Vargas P, Carpi N, et al. Perinuclear Arp2/3-driven actin polymerization  
484 enables nuclear deformation to facilitate cell migration through complex environments. *Nat*  
485 *Commun*. 2016;7:10997.
- 486 18. Vargas P, Maiuri P, Bretou M, et al. Innate control of actin nucleation determines two  
487 distinct migration behaviours in dendritic cells. *Nat Cell Biol*. 2016;18(1):43-53.
- 488 19. Semba H, Takeda N, Isagawa T, et al. HIF-1alpha-PDK1 axis-induced active glycolysis  
489 plays an essential role in macrophage migratory capacity. *Nat Commun*. 2016;7:11635.

- 490 20. Vogel S, Wottawa M, Farhat K, et al. Prolyl Hydroxylase Domain (PHD) 2 affects cell  
491 migration and F-actin formation via RhoA/ROCK-dependent cofilin phosphorylation. *Journal*  
492 *of Biological Chemistry*. 2010;jbc. M110. 132985.
- 493 21. Choi HJ, Sanders TA, Tormos KV, et al. ECM-dependent HIF induction directs  
494 trophoblast stem cell fate via LIMK1-mediated cytoskeletal rearrangement. *PloS one*.  
495 2013;8(2):e56949.
- 496 22. Huang C, Qian SL, Sun LY, Cheng B. Light-Emitting Diode Irradiation (640 nm)  
497 Regulates Keratinocyte Migration and Cytoskeletal Reorganization Via Hypoxia-Inducible  
498 Factor-1 $\alpha$ . *Photomedicine and laser surgery*. 2016;34(8):313-320.
- 499 23. Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med*.  
500 2013;369(21):2063.
- 501 24. Singh RP, Franke K, Kalucka J, et al. HIF prolyl hydroxylase 2 (PHD2) is a critical  
502 regulator of hematopoietic stem cell maintenance during steady-state and stress. *Blood*.  
503 2013;121(26):5158-5166.
- 504 25. Stadtfeld M, Graf T. Assessing the role of hematopoietic plasticity for endothelial and  
505 hepatocyte development by non-invasive lineage tracing. *Development*. 2005;132(1):203-213.
- 506 26. Gruber M, Hu C-J, Johnson RS, Brown EJ, Keith B, Simon MC. Acute postnatal  
507 ablation of Hif-2 $\alpha$  results in anemia. *Proc Natl Acad Sci U S A*. 2007;104(7):2301-2306.
- 508 27. Kiers D, Wielockx B, Peters E, et al. Short-Term Hypoxia Dampens Inflammation in  
509 vivo via Enhanced Adenosine Release and Adenosine 2B Receptor Stimulation. *EBioMedicine*.  
510 2018;33:144-156.
- 511 28. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-  
512 specific disease provoked by systemic autoimmunity. *Cell*. 1996;87(5):811-822.
- 513 29. Vargas P, Terriac E, Lennon-Dumenil AM, Piel M. Study of cell migration in  
514 microfabricated channels. *J Vis Exp*. 2014(84):e51099.

- 515 30. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for  
516 biological-image analysis. *Nat Methods*. 2012;9(7):676-682.
- 517 31. Vargas P, Chabaud M, Thiam H-R, Lankar D, Piel M, Lennon-Dumenil A-M. Study of  
518 dendritic cell migration using micro-fabrication. *Journal of immunological methods*.  
519 2016;432:30-34.
- 520 32. Ufer F, Vargas P, Engler JB, et al. Arc/Arg3.1 governs inflammatory dendritic cell  
521 migration from the skin and thereby controls T cell activation. *Science immunology*.  
522 2016;1(3):eaaf8665-eaaf8665.
- 523 33. Sáez PJ, Vargas P, Shoji KF, Harcha PA, Lennon-Duménil A-M, Sáez JC. ATP  
524 promotes the fast migration of dendritic cells through the activity of pannexin 1 channels and  
525 P2X7 receptors. *Sci Signal*. 2017;10(506):eaah7107.
- 526 34. Barbier L, Saez PJ, Attia R, et al. Myosin II Activity Is Selectively Needed for  
527 Migration in Highly Confined Microenvironments in Mature Dendritic Cells. *Front Immunol*.  
528 2019;10:747.
- 529 35. Dai Z, Li M, Wharton J, Zhu MM, Zhao Y-Y. PHD2 deficiency in endothelial cells and  
530 hematopoietic cells induces obliterative vascular remodeling and severe pulmonary arterial  
531 hypertension in mice and humans through HIF-2 $\alpha$ . *Circulation*. 2016;133(24):2447.
- 532 36. Sadiku P, Willson JA, Dickinson RS, et al. Prolyl hydroxylase 2 inactivation enhances  
533 glycogen storage and promotes excessive neutrophilic responses. *J Clin Invest*.  
534 2017;127(9):3407-3420.
- 535 37. Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. *The Journal*  
536 *of cell biology*. 2009:jcb.200909003.
- 537 38. Cramer LP. Forming the cell rear first: breaking cell symmetry to trigger directed cell  
538 migration. *Nature cell biology*. 2010;12(7):628.

- 539 39. Saez PJ, Barbier L, Attia R, Thiam HR, Piel M, Vargas P. Leukocyte Migration and  
540 Deformation in Collagen Gels and Microfabricated Constrictions. *Methods Mol Biol.*  
541 2018;1749:361-373.
- 542 40. Mietke A, Otto O, Girardo S, et al. Extracting Cell Stiffness from Real-Time  
543 Deformability Cytometry: Theory and Experiment. *Biophys J.* 2015;109(10):2023-2036.
- 544 41. Mokbel M, Mokbel D, Mietke A, et al. Numerical Simulation of Real-Time  
545 Deformability Cytometry To Extract Cell Mechanical Properties. *ACS Biomaterials Science &*  
546 *Engineering.* 2017;3(11):2962-2973.
- 547 42. Prathivadhi-Bhayankaram SV, Ning J, Mimplitz M, et al. Chemotherapy impedes in  
548 vitro microcirculation and promotes migration of leukemic cells with impact on metastasis.  
549 *Biochemical and biophysical research communications.* 2016;479(4):841-846.
- 550 43. Ekpenyong AE, Toepfner N, Fiddler C, et al. Mechanical deformation induces  
551 depolarization of neutrophils. *Science advances.* 2017;3(6):e1602536.
- 552 44. Elks PM, van Eeden FJ, Dixon G, et al. Activation of hypoxia-inducible factor-1 $\alpha$  (Hif-  
553 1 $\alpha$ ) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in  
554 a zebrafish inflammation model. *Blood.* 2011;118(3):712-722.
- 555 45. Alarcon-Barrera JC, von Hegedus JH, Brouwers H, et al. Lipid metabolism of  
556 leukocytes in the unstimulated and activated states. *Anal Bioanal Chem.* 2020;412(10):2353-  
557 2363.
- 558 46. Tzeng HT, Chyuan IT, Chen WY. Shaping of Innate Immune Response by Fatty Acid  
559 Metabolite Palmitate. *Cells.* 2019;8(12).
- 560 47. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a  
561 knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad*  
562 *Sci U S A.* 2005;102(43):15545-15550.



- 563 48. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved  
564 in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet.*  
565 2003;34(3):267-273.
- 566 49. Singh RP, Grinenko T, Ramasz B, et al. Hematopoietic Stem Cells but Not Multipotent  
567 Progenitors Drive Erythropoiesis during Chronic Erythroid Stress in EPO Transgenic Mice.  
568 *Stem Cell Reports.* 2018;10(6):1908-1919.
- 569 50. Schodel J, Oikonomopoulos S, Ragoussis J, Pugh CW, Ratcliffe PJ, Mole DR. High-  
570 resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood.*  
571 2011;117(23):e207-217.
- 572 51. Ditzel HJ. The K/BxN mouse: a model of human inflammatory arthritis. *Trends in*  
573 *Molecular Medicine.* 2004;10(1):40-45.
- 574 52. Monach PA, Mathis D, Benoist C. The K/BxN arthritis model. *Curr Protoc Immunol.*  
575 2008;Chapter 15:Unit 15 22.
- 576 53. Lämmermann T, Bader BL, Monkley SJ, et al. Rapid leukocyte migration by integrin-  
577 independent flowing and squeezing. *Nature.* 2008;453(7191):51.
- 578 54. Afonso PV, Janka-Junttila M, Lee YJ, et al. LTB4 is a signal-relay molecule during  
579 neutrophil chemotaxis. *Developmental cell.* 2012;22(5):1079-1091.
- 580 55. Lämmermann T, Afonso PV, Angermann BR, et al. Neutrophil swarms require LTB4  
581 and integrins at sites of cell death in vivo. *Nature.* 2013;498(7454):371.
- 582 56. Reátegui E, Jalali F, Khankhel AH, et al. Microscale arrays for the profiling of start and  
583 stop signals coordinating human-neutrophil swarming. *Nature biomedical engineering.*  
584 2017;1(7):0094.
- 585 57. Renkawitz J, Sixt M. Mechanisms of force generation and force transmission during  
586 interstitial leukocyte migration. *EMBO Rep.* 2010;11(10):744-750.

- 587 58. Yamada KM, Sixt M. Mechanisms of 3D cell migration. *Nat Rev Mol Cell Biol.*  
588 2019;20(12):738-752.
- 589 59. Toyjanova J, Flores-Cortez E, Reichner JS, Franck C. Matrix confinement plays a  
590 pivotal role in regulating neutrophil-generated tractions, speed, and integrin utilization. *J Biol*  
591 *Chem.* 2015;290(6):3752-3763.
- 592 60. Heemskerk N, Schimmel L, Oort C, et al. F-actin-rich contractile endothelial pores  
593 prevent vascular leakage during leukocyte diapedesis through local RhoA signalling. *Nat*  
594 *Commun.* 2016;7:10493.
- 595 61. Filippi MD. Neutrophil transendothelial migration: updates and new perspectives.  
596 *Blood.* 2019;133(20):2149-2158.
- 597 62. Hind LE, Vincent WJ, Huttenlocher A. Leading from the Back: The Role of the Uropod  
598 in Neutrophil Polarization and Migration. *Dev Cell.* 2016;38(2):161-169.
- 599 63. Sit ST, Manser E. Rho GTPases and their role in organizing the actin cytoskeleton. *J*  
600 *Cell Sci.* 2011;124(Pt 5):679-683.
- 601 64. Jennings RT, Strengert M, Hayes P, et al. RhoA determines disease progression by  
602 controlling neutrophil motility and restricting hyperresponsiveness. *Blood.*  
603 2014;123(23):3635-3645.
- 604

Figure 1

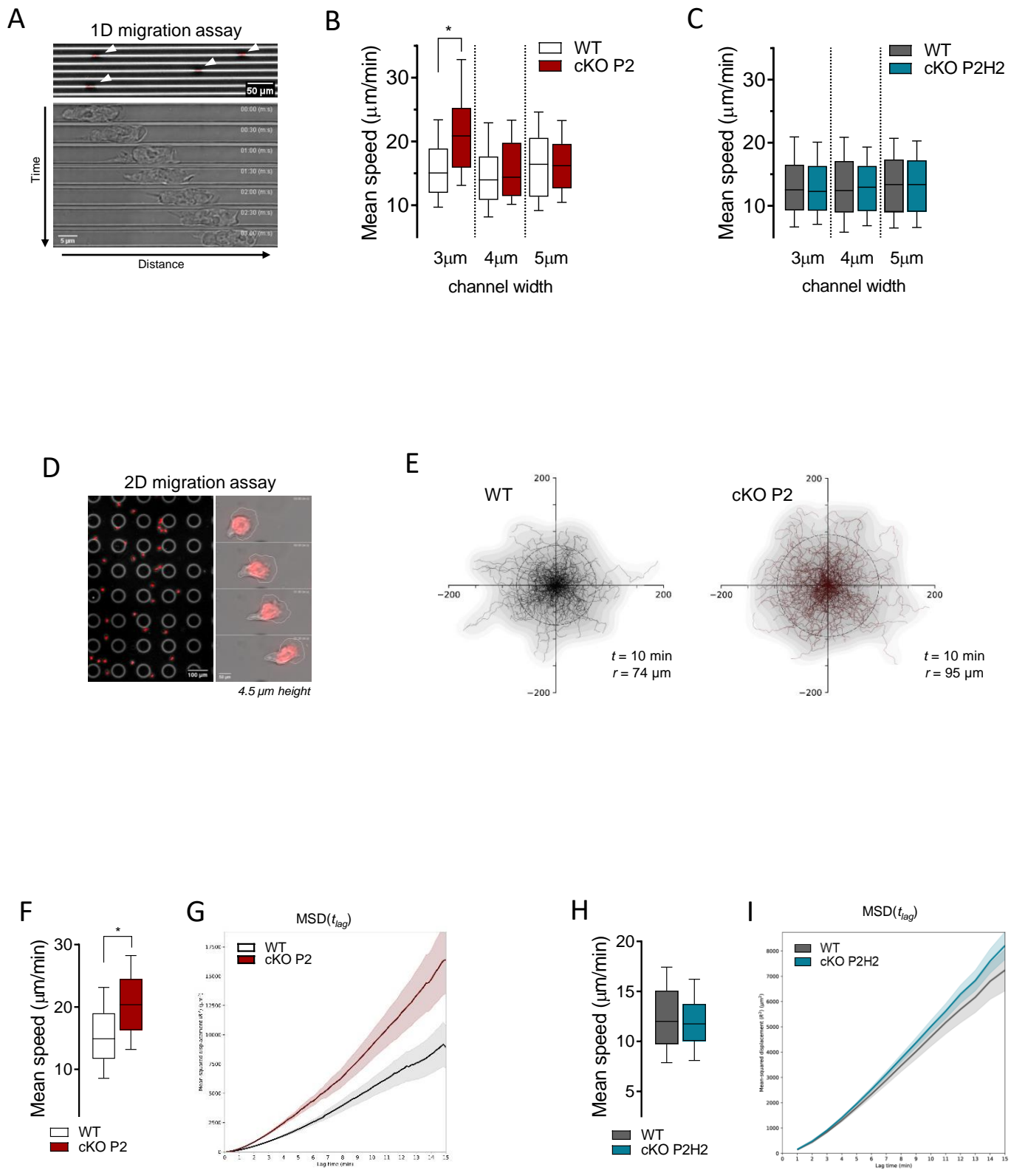


Figure 2

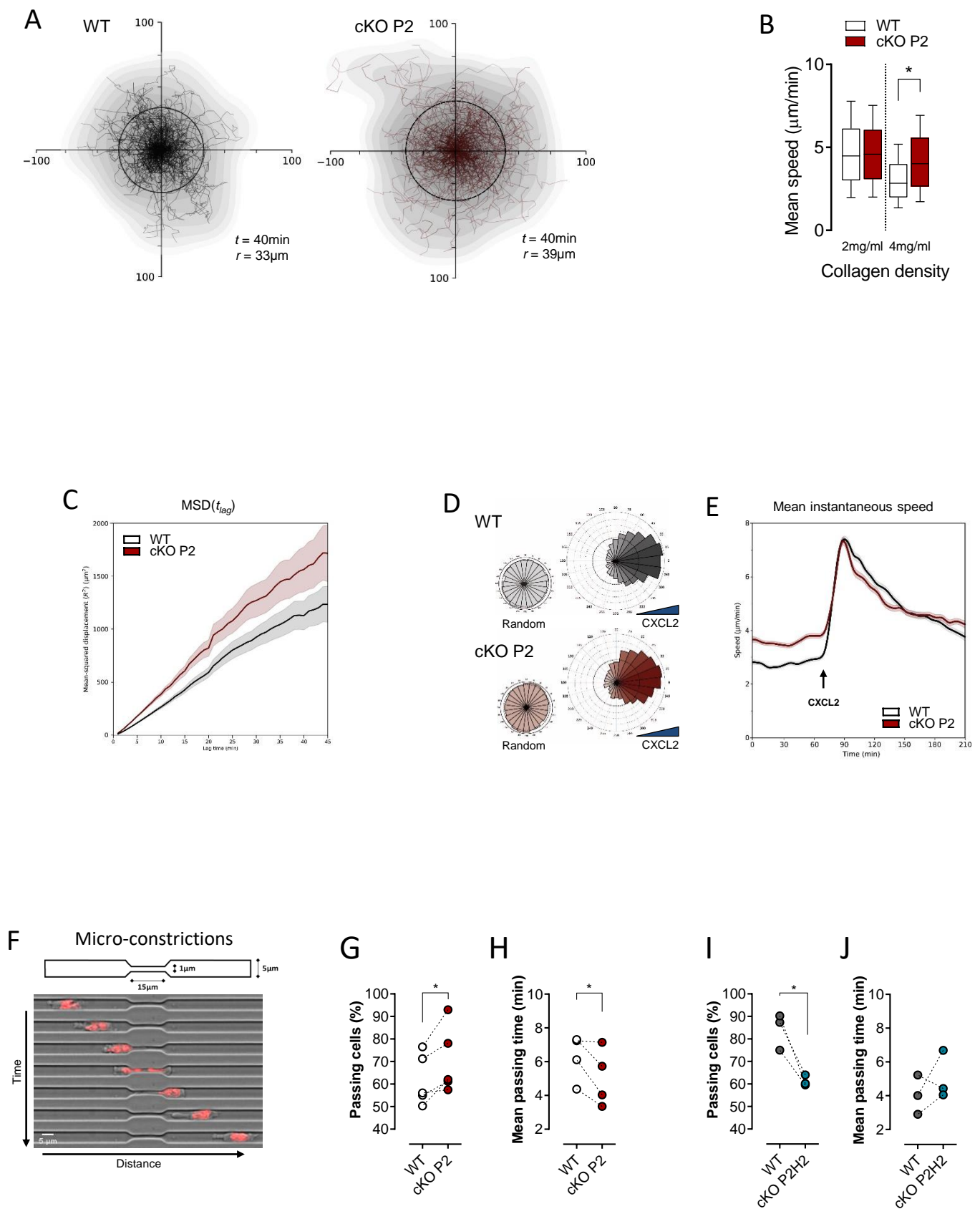


Figure 3

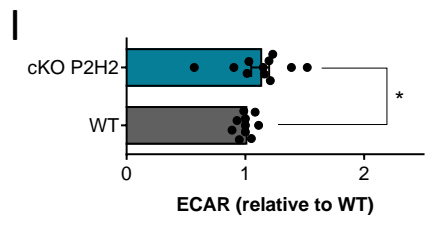
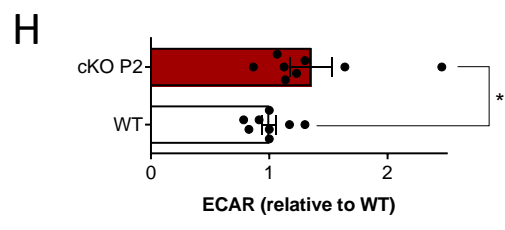
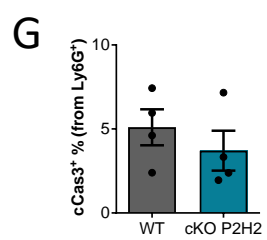
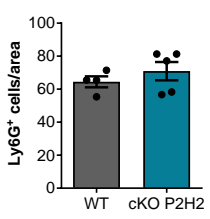
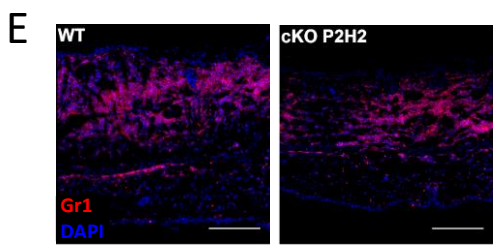
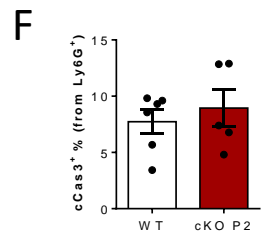
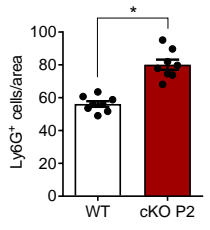
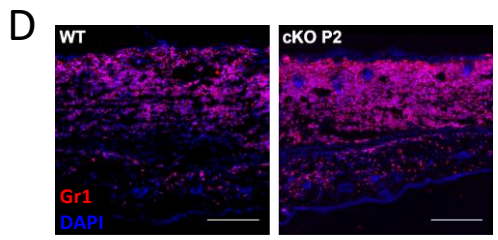
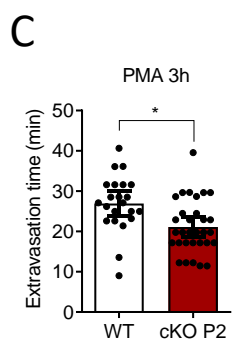
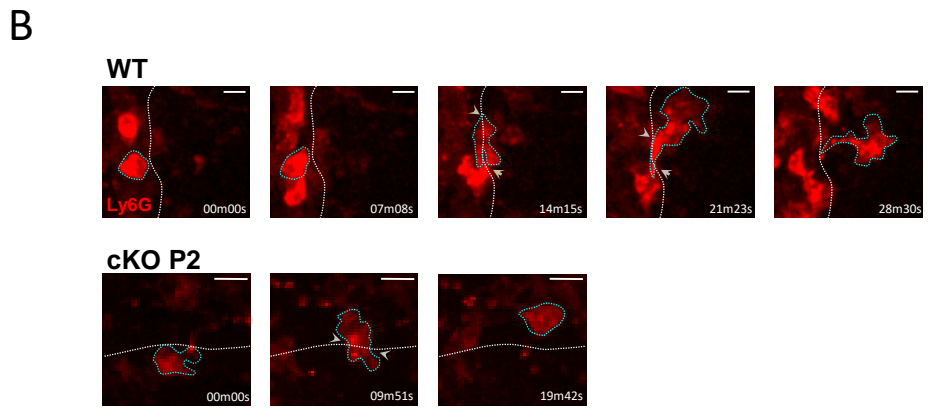
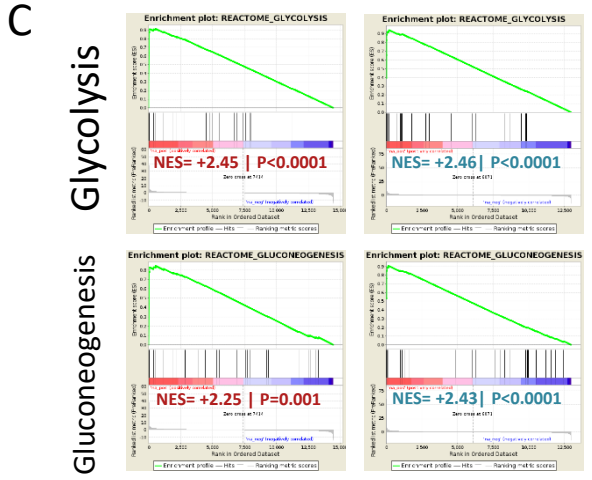
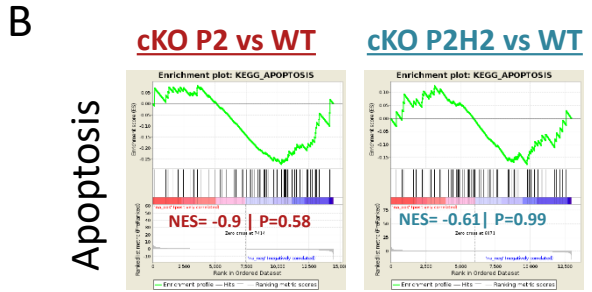
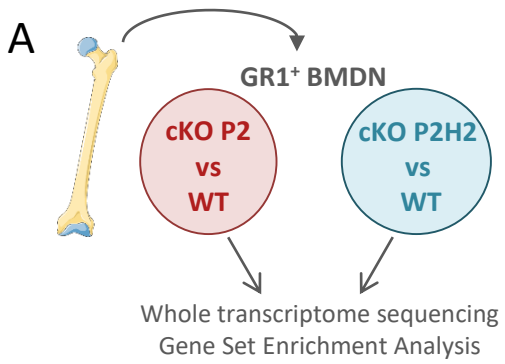
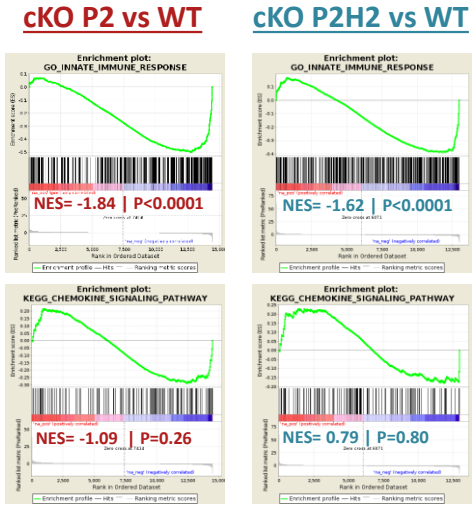


Figure 4



Immune response



Cytoskeleton  
Function  
Structure

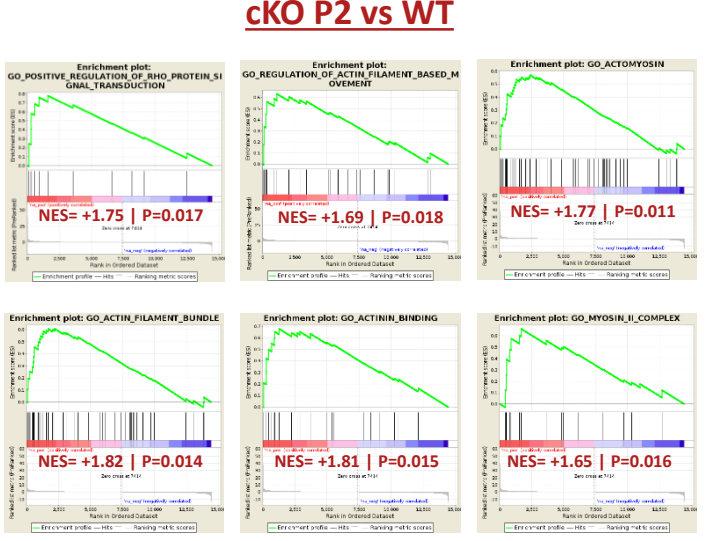






Figure 6

