

1 **Membrane receptor MerTK is a newly identified transcriptional  
2 regulator that associates to chromatin as nanoclusters during human  
3 DC differentiation**

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## 26 Abstract

27 MerTK is a transmembrane receptor tyrosine kinase (RTK) mainly expressed in dendritic  
28 cells (DCs) and macrophages where it plays an important role in immunotolerance, but  
29 also in activating oncogenic signalling pathways. Albeit MerTK is exploited as clinical  
30 target in cancer and auto-immune disorders, the mechanisms that regulate its diverse  
31 functions are poorly understood. Here, we identified a remarkably high pool of the full  
32 receptor in the nucleus of human DCs. Nuclear translocation was ligand-dependent.  
33 Importantly, MerTK nuclear levels correlated to DC differentiation and were  
34 spatiotemporally regulated by the transmembrane receptor LRP-1. Using dual-colour  
35 super-resolution nanoscopy we discovered that nuclear MerTK forms nanoclusters,  
36 whose strength strongly depends on chromatin accessibility during DC differentiation.  
37 We finally revealed high transcription capacity of MerTK. Overall, our work indicates  
38 that nuclear MerTK acts as a transcription factor regulating DC differentiation, thus  
39 implicating for the first time a physiological function for RTK nuclear translocation in  
40 immunity.

41 **Introduction**

42 RTKs comprise a family of cell-surface receptors key in regulating essential cellular  
43 processes such as growth, differentiation, survival and migration (Lemmon and  
44 Schlessinger, 2010). Structurally highly conserved, these receptors contain an  
45 extracellular domain, a single transmembrane domain, and an intracellular kinase domain.  
46 Ligand binding at the extracellular domain activates the receptor by inducing homo-  
47 dimerization and subsequent auto-phosphorylation of tyrosine residues in the cytoplasmic  
48 tail that initiate downstream signalling cascades (Hubbard, 1999; Li and Hristova, n.d.).  
49 MerTK is a member of the RTK family that regulates an intriguingly broad range of  
50 seemingly unrelated cellular processes, including apoptosis, migration, transcription  
51 (Cummings et al., 2013; Graham et al., 2014; Linger et al., 2008), and immunotolerance  
52 (Cabezón et al., 2015; Camenisch et al., 1999; Lu and Lemke, 2001; Rothlin et al., 2007;  
53 Rothlin and Lemke, 2010; Scott et al., 2001; Wallet et al., 2008). Physiologically, MerTK  
54 is mainly expressed on the surface of macrophages and DCs (Behrens et al., 2003), where  
55 it plays a role in phagocytosis of apoptotic cells (Scott et al., 2001) and in downregulating  
56 the secretion of pro-inflammatory cytokines (Sen et al., 2007). Loss of MerTK function  
57 and of its family members Tyro-3 and Axl (TAM family) leads to inflammation and  
58 increased susceptibility for auto-immune disorders (Lu and Lemke, 2001; Rothlin and  
59 Lemke, 2010). In contrast, ectopic or increased expression of MerTK has been found in  
60 a wide variety of cancers where it activates oncogenic signalling pathways leading to  
61 increased cell survival, invasion, and therapy resistance (Cummings et al., 2013; Graham  
62 et al., 2014).

63 Due to its broad involvement in cancer and auto-immune disorders, MerTK is  
64 being increasingly exploited as a potential clinical target. Multiple reports have  
65 demonstrated the effectiveness and specificity of MerTK inhibition in tumour

66 suppression (Brandao et al., 2013; Cook et al., 2013; Crittenden et al., 2016; Cummings  
67 et al., 2015; Kim et al., 2017). In the context of immunity, a recent study in human DCs  
68 showed that MerTK is highly upregulated upon several days of tolerogenic treatment with  
69 Dexamethasone (Cabezón et al., 2015). These so-called tolerogenic DCs suppress both T  
70 cell expansion and pro-inflammatory cytokine production by T cells (Cabezón et al.,  
71 2015), a process that is regulated by MerTK. Several ongoing clinical trials indeed exploit  
72 the immune tempering function of MerTK, among other immunosuppressive  
73 mechanisms, by injecting tolerogenic DCs into patients in order to battle different auto-  
74 immune disorders such as diabetes type I (Giannoukakis, 2013), Rheumatoid Arthritis  
75 (Bell et al., 2017; Benham et al., 2015) or Crohn's disease (Jauregui-Amezaga et al.,  
76 2015). Surprisingly, despite the evident clinical relevance of MerTK signalling, very little  
77 is known on the molecular mechanisms of action by which this receptor is able to  
78 accomplish its broad range of functions.

79           Although the function of MerTK has been classically associated to its expression  
80 at the plasma membrane, a recent study on human tolerogenic DCs identified an  
81 abnormally large pool of the receptor located intracellularly and accounting for as much  
82 as 40% of its total expression levels (Cabezón et al., 2015). However, the subcellular  
83 location as well as function of this intracellular MerTK pool has remained completely  
84 elusive. We hypothesized that the existence of several pools of the receptor with distinct  
85 subcellular localizations might be important in defining its functional diversity. We thus  
86 employed biochemical tools together with advanced optical imaging techniques,  
87 including super-resolution microscopy, to investigate the spatial distribution of MerTK  
88 in immunogenic and tolerogenic human DCs. Remarkably, we found that intracellular  
89 MerTK is mainly located in the nucleus and that its degree of nuclear accumulation is  
90 strictly related to DC differentiation. Moreover, once in the nucleus, MerTK associates

91 to chromatin and it is capable to induce transcription. As a whole, our results indicate that  
92 aside from its well-established role on the cell membrane, the residence of MerTK in the  
93 nucleus constitutes a physiological relevant mechanism for dendritic cells, functioning as  
94 a potential genomic regulator during DC differentiation. Given the involvement of  
95 MerTK in both auto-immunity and cancer, our results might have impact on the broad  
96 implementation of MerTK for clinical therapy purposes.

97

## 98 **Results**

### 99 **MerTK is found both on the membrane and in the nucleus of tolerogenic DCs**

100 Previous studies by Cabezon et al. (Cabezón et al., 2015) showed that MerTK is highly  
101 upregulated in immature tolerogenic DCs upon several days of tolerogenic treatment with  
102 the glucocorticoid Dexamethasone (Dex). We first confirmed by flow cytometry that  
103 these Dex-treated immature DCs, referred to as iDex, highly express MerTK on their  
104 membrane, as opposed to immunogenic immature DCs (iDCs) (Fig. 1A). To elucidate the  
105 spatial organization of the receptor on the cell membrane at the single cell level, we  
106 performed super-resolution, stimulated emission depletion (STED) nanoscopy imaging.  
107 With a spatial resolution of ~100nm, we identified well-separated fluorescent spots of  
108 MerTK homogeneously distributed across the plasma membrane of iDex cells (Fig. 1B).  
109 These spots correspond to small MerTK nanoclusters containing on average 3 and up to  
110 10 receptors (see materials and methods) (Fig. 1C), and having sizes ~120nm (Fig. 1D).  
111 This kind of organization is in good agreement with the general consensus that  
112 nanoclusters are the functional unit for many immunoreceptors on the plasma membrane  
113 (Akiyama et al., 2015; Garcia-Parajo et al., 2014; Torreno-Pina et al., 2016, 2014; van  
114 Zanten et al., 2009).

115           Recent flow cytometry studies performed on human DCs showed that ~40% of  
116           MerTK resides intracellularly, both under immunogenic as well as tolerogenic conditions  
117           (Cabezón et al., 2015). To identify the location of this intracellular pool we performed  
118           confocal imaging of MerTK together with different organelle markers on both iDCs and  
119           iDex cells. To first exclude the possibility that the intracellular pool of MerTK  
120           corresponds to receptors targeted for degradation, we labelled MerTK and lysosomes. A  
121           clear exclusion of MerTK from the lysosome compartment was observed ([Supplementary](#)  
122           Fig. 1). Interestingly, we found that the MerTK intracellular pool almost entirely resides  
123           inside the nucleus ([Fig. 1E](#)). To validate the specificity of the antibodies used for imaging,  
124           we further probed MerTK using two other antibodies raised against different extracellular  
125           epitopes from different manufacturers, and obtained the same nuclear distribution  
126           ([Supplementary Figs. 2A,B](#)). A fourth antibody against an intracellular epitope of MerTK  
127           also gave the same spatial distribution ([Fig. 1F](#)), indicating that both intracellular and  
128           extracellular parts of the protein exhibit nuclear localization. This result also suggests that  
129           the nuclear MerTK pool observed by us, does not correspond to a previously reported  
130           soluble isoform consisting of the extracellular domain of the protein (Sather et al., 2007),  
131           nor to its intracellular counterpart that occurs after proteolytic cleavage. Quantification  
132           of the amount of nuclear MerTK in both iDCs and iDex (3 different donors, each) from  
133           fluorescent images shows that iDexs exhibit a more pronounced MerTK nuclear  
134           accumulation as compared to iDCs ([Fig. 1G](#)). Nevertheless, this increase (~20%) was  
135           much more modest as compared to the three-fold increase in the expression level detected  
136           at the cell membrane ([Fig. 1A](#)).

137           To further validate the physiological relevance of our results and to rule out  
138           potential artefacts caused by the *in-vitro* differentiation of the tolerogenic DCs, we  
139           isolated immune cells with a tolerogenic phenotype directly from the tumour

140 environment. Also, in these cells, MerTK nuclear distribution was clearly observed  
141 ([Supplementary Fig. 2C](#)). Nuclear localization was detected in the monocytic cell line  
142 THP-1 as well ([Supplementary Fig. 2D](#)). Altogether, these results show a remarkable high  
143 MerTK nuclear localization in different immune cells: cells from the THP-1 cell line, *in-*  
144 *vitro* monocytic derived DCs, and directly isolated immune cells.

145

146 **Nuclear MerTK levels strictly correlate with DC differentiation**

147 Membrane expression of MerTK on human DCs was previously shown to depend on  
148 tolerogenic treatment with Dex (Cabezón et al., 2015). Indeed, surface MerTK is absent  
149 in monocytes and only steeply increases in the first two days after tolerogenic treatment,  
150 in contrast to cells equally differentiated in the absence of Dex where membrane  
151 expression is always minimal (Cabezón et al., 2015). We therefore enquired whether the  
152 same dependence would hold for nuclear MerTK (*n*MerTK) upon Dex treatment. For  
153 this, we permeabilized the cells and quantified, by confocal microscopy, the amount of  
154 *n*MerTK during each of the seven days of differentiation from monocytes into  
155 iDCs/iDexs. In contrast to the strong effect that Dex had on the expression levels of  
156 MerTK on the cell membrane, tolerogenic treatment had only a minor influence on the  
157 amount of *n*MerTK, as nuclear expression levels were comparable in the presence or  
158 absence of Dex ([Fig. 2A](#)) with only a modest increase in iDex at day four (last time point  
159 in [Fig. 2A](#), and [Fig. 1G](#)). Remarkably, a strong correlation between the amount of  
160 *n*MerTK and the stage of DC differentiation was observed, reaching maximum levels at  
161 day 0, the moment when monocytes transition into early DCs. Moreover, *n*MerTK  
162 localization occurred gradually, starting with a low overall expression in monocytes (day  
163 -2), massive expression increase and distribution throughout the entire cytoplasm (day -  
164 1) and specific nuclear accumulation around day 0 ([Fig. 2B](#)). Nuclear accumulation then

165 persisted as a function of DC differentiation and led to a well-defined distribution (day 4)

166 where almost all the intracellular MerTK resides in the nucleus (Fig. 2B).

167 To further demonstrate that this increase in *n*MerTK expression is specific to DC

168 differentiation rather than to days of *in-vitro* culturing, we assessed the effect of the

169 differentiation cocktail (cytokines IL-4 and GM-CSF that together facilitate the

170 differentiation of monocytes into DCs (de Vries et al., 2005)) on the amount of *n*MerTK.

171 First, we used different doses of IL-4 to transition monocytes into newly DCs (at day 0).

172 As expected, the higher the dose of this differentiating cytokine (within the physiological

173 relevant range of DC differentiation), the more MerTK accumulates into the nucleus (Fig.

174 2C), indicating a correlation between differentiation and *n*MerTK. Second, we

175 administered the full cocktail to THP-1 monocytic cells that had been previously cultured

176 for several cell cycles to induce their differentiation in to DC-like cells (Berges et al.,

177 2005; Guo et al., 2012). Also in these conditions a strong increase in *n*MerTK was

178 observed (Fig. 2D), along with a clear change in cell morphology indicative of a DC-like

179 phenotype (Supplementary Fig. 3A-C). Altogether these data demonstrate that the degree

180 of *n*MerTK expression strictly correlates with DC differentiation, reaching its maximum

181 at the transition point from monocytes into early DCs.

182 Over the last decade, a few reports have indicated the presence of some RTKs in

183 the nucleus as a malignant side effect resulting from its overexpression in tumour cells

184 (Brand et al., 2012; Wang and Hung, 2012; Wells and Marti, 2002a). To investigate

185 whether MerTK would follow a similar pattern, we overexpressed the receptor in

186 different tumour cell lines. HeLa cells transfected with MerTK showed a membrane

187 expression profile similar to that of DCs with the presence of small nanoclusters

188 (Supplementary Fig. 3D). Nevertheless, this aberrant expression did not result in MerTK

189 nuclear translocation (Supplementary Fig. 3E). Likewise, HEK-293 cells which

190 endogenously express MerTK, do not exhibit *n*MerTK ([Supplementary Fig. 3F](#)), neither  
191 does its overexpression induce nuclear translocation ([Supplementary Fig. 3G](#)). Thus, in  
192 contrast to other RTKs, our data show that *n*MerTK is not the result of aberrant or  
193 overexpression of the receptor, but is rather a trait exclusively reserved for immune cells  
194 (THP-1, several types of DCs and Jurkat T cells (Migdall-Wilson et al., 2012)),  
195 suggesting a physiological role for *n*MerTK in immune cells.

196

### 197 **Binding of ligand ProS induces *n*MerTK translocation**

198 Trafficking from the membrane into the nucleus has previously been reported for several  
199 RTKs (Chen and Hung, 2015; Wells and Marti, 2002a), amongst which the epidermal  
200 growth factor receptor (EGFR) has received most attention (Wang et al., 2010; Wang and  
201 Hung, 2012). In these cases, nuclear translocation was found to be ligand induced  
202 (Carpenter and Liao, 2009; Lin et al., 2001). This prompted us to explore the role of the  
203 MerTK ligand ProS (Lemke and Rothlin, 2008; Stitt et al., 1995) in *n*MerTK translocation  
204 by directly imaging the receptor and ProS on individual iDex cells. Even though GAS6  
205 is also a well-described ligand for MerTK (Chen et al., 1997; Nagata et al., 1996), we  
206 focused on ProS as it has been previously described to be the main ligand involved in  
207 immunoregulation by human DCs (Carrera Silva et al., 2013). A strong colocalization  
208 between MerTK and ProS was observed intracellularly ([Fig. 3A](#)) together with the  
209 presence of multiple receptor-ligand complexes associated to the nuclear envelope (NE)  
210 (orange arrows in [Fig. 3B](#)). To quantify the degree of colocalization between MerTK and  
211 ProS at different subcellular regions, we segmented the cell images into periphery (mostly  
212 membrane), cytoplasm, and NE bound. At the cell periphery, colocalization is low  
213 suggesting that MerTK internalization quickly follows after ProS binding ([Fig. 3C](#)). In

214 strong contrast, colocalization markedly increases towards the nucleus, consistent with  
215 ligand induced intracellular MerTK trafficking.

216 To further demonstrate the involvement of ProS in *n*MerTK translocation we  
217 performed similar imaging experiments on newly differentiated DCs where *n*MerTK  
218 levels were found to be maximum (day 0, [Fig. 2A](#)). We hypothesized that more ProS  
219 would be found in the nucleus of the cells at day 0 as compared to day 4 (iDex cells, [Fig.](#)  
220 [3A–C](#)). We found a 2.5-fold increase of nuclear ProS at day 0 compared to day 4 ([Fig.](#)  
221 [3D](#)), supporting the notion that ProS indeed plays an important role in facilitating  
222 *n*MerTK trafficking. Since DCs require the presence of human serum (HS) in their growth  
223 medium and HS naturally contains high levels of ProS, it is not feasible to fully deprive  
224 the cells of ProS to further investigate its direct effect on *n*MerTK translocation. As an  
225 alternative, we cultured DCs in the presence of highly reduced HS levels, and compared  
226 *n*MerTK accumulation to cells cultured in the same reduced serum conditions but with  
227 the extra addition of soluble ProS. A significant increase in the amount of *n*MerTK was  
228 observed at these higher levels of ProS ([Fig. 3E](#)), further strengthening our findings that  
229 *n*MerTK translocation is ligand dependent.

230

### 231 **The endocytic receptor LRP-1 facilitates *n*MerTK translocation**

232 We showed that MerTK expression is upregulated at two different stages during the  
233 differentiation of monocytes into iDex. First, at day 0, where upregulation is accompanied  
234 with a high localization of the receptor in the nucleus ([Fig. 2A](#)) and second, on fully  
235 differentiated iDex, where upregulated MerTK is mostly associated to the plasma  
236 membrane ([Fig. 1A](#) and Ref. 13). Although we showed that *n*MerTK translocation is  
237 facilitated by ProS, serum levels of ProS are constant through the entire DC  
238 differentiation process and as such, the ligand on its own is not likely to fully determine

239 the spatial destination of the receptor. We thus hypothesized that MerTK requires an  
240 additional factor chaperoning its shuttling towards the nucleus at day 0 and that moreover,  
241 this factor must be lacking (or downregulated) in fully differentiated iDex, where MerTK  
242 remains largely membrane associated (Fig. 1A and Ref. 13). An interesting candidate for  
243 this differential spatial regulation is the endocytic receptor LRP-1, which is known to  
244 form a complex with Axl to facilitate internalization (Subramanian et al., 2014). Axl and  
245 MerTK are close relatives within the TAM family, making it conceivable that MerTK  
246 and LRP-1 can interact in a similar manner. Furthermore, LRP-1 has been reported to  
247 play a role in the shuttling of soluble environmental factors into the nucleus (Chaumet et  
248 al., 2015). To elucidate whether LRP-1 plays a shuttling role for MerTK, we performed  
249 intracellular dual colour confocal imaging of both MerTK and LRP-1. A remarkably  
250 strong colocalization between both receptors was observed (Fig. 4A) with multiple  
251 receptor complexes associated to the NE (orange arrows in Fig. 4B and quantification  
252 over multiple cells in Fig. 4C).

253 We then quantified the amount of nuclear ( $n$ )LRP-1 as a function of DC  
254 differentiation. Interestingly,  $n$ LRP-1 expression follows a similar trend as to  $n$ MerTK,  
255 i.e., being highest at day 0, and decreasing steadily as a function of DC differentiation  
256 (Fig. 4D). However, unlike MerTK whose total expression increases again towards the  
257 final stage of differentiation (mostly located on the membrane), the total expression levels  
258 of LRP-1 remain low on fully differentiated iDex (Supplementary Fig. 4A). These results  
259 thus suggest that LRP-1 might play a role in the spatial partitioning of MerTK,  
260 determining whether nuclear translocation or membrane expression takes place.

261 In a model in which LRP-1 acts as a chaperone in bringing MerTK from the  
262 membrane to the nucleus, one would expect to find a positive correlation between the  
263 expression levels of both proteins in the nucleus. Taking advantage of naturally occurring

264 cell to cell variability, we quantified the levels of *n*MerTK and *n*LRP-1 at the single cell  
265 level over multiple cells. Indeed, as suspected, a positive correlation between the  
266 translocation of both receptors was obtained (Fig. 4E). To inquire which receptor is  
267 leading and which receptor is following in this correlated translocation, we stimulated  
268 LRP-1 by adding one of its many ligands, RAP, in the medium. RAP-stimulation  
269 significantly increased nuclear LRP-1 translocation (Supplementary Fig. 4B) and most  
270 importantly, it also led to increased *n*MerTK translocation (Fig. 4F), again pointing  
271 towards a facilitating role of LRP-1 in the shuttling of MerTK into the nucleus. Overall,  
272 our results strongly suggest that LRP-1 plays a major role as chaperone molecule in  
273 regulating the sub-cellular spatial partitioning of MerTK, either to the nucleus or to the  
274 membrane.

275

### 276 ***n*MerTK is associated to chromatin, preferentially in an open conformation**

277 The intriguing findings of the existence of a *n*MerTK population in immune cells  
278 prompted us to assess its specific nuclear location at the nanoscale, as well as its potential  
279 role. For this, we first separated day 0 DCs and DC-like THP-1 cells that both highly  
280 express *n*MerTK in different cellular fractions: the cytoplasm, the soluble part inside the  
281 nucleus, and the chromatin-bound fraction (Wang et al., 2015). These fractions were  
282 subsequently analysed by Western blotting with an antibody against MerTK. MerTK was  
283 found in all three fractions in both DCs and DC-like THP-1 cells, including the  
284 chromatin-bound fraction (Supplementary Fig 5A). This result suggests that *n*MerTK  
285 could play a role in gene expression regulation.

286 To investigate the spatial relationship between *n*MerTK and chromatin at the  
287 molecular level, we used dual colour Stochastic Optical Reconstruction Microscopy  
288 (STORM) following the approach of Ricci et al (Ricci et al., 2015) (Supplementary Fig

289 5B,C). This super-resolution technique allowed us to identify individual fluorescently-  
290 labelled *n*MerTK and histone molecules within the crowded environment of the nucleus  
291 with a localization precision of about 20 nm (Fig. 5A). In mammalian cells, the nuclear  
292 periphery is enriched in condensed heterochromatin, generally associated with  
293 transcriptional repression (Dekker and Misteli, 2015). Several studies have further  
294 demonstrated a direct link between the association of chromatin to the nuclear lamina and  
295 gene silencing (Finlan et al., 2008; Guelen et al., 2008; Kosak et al., 2002; Reddy et al.,  
296 2008). Consistent with these published results, our STORM images showed the  
297 condensed heterochromatin as a dense ring at the edge of the nucleus (Fig. 5A).  
298 Interestingly, this ring was mostly deprived of *n*MerTK (Fig. 5A, upper right panel).  
299 Remarkably, *n*MerTK was observed in the central nuclear region where the chromatin is  
300 much less dense (euchromatin) (Fig. 5A, lower right panel). In these regions, we also  
301 observed elongated structures composed of *n*MerTK and histones (Fig. 5A, pink dotted  
302 lines) that resemble a configuration where the nucleosomes are well-separated, DNA  
303 occupancy is low and chromatin is accessible (Ricci et al., 2015). The strong localization  
304 of *n*MerTK to nuclear regions where DNA is in an accessible configuration together with  
305 its clear exclusion from dense heterochromatin regions suggest that *n*MerTK might  
306 interact with active genomic regions.

307 To further investigate this possibility, we quantified the degree of spatial  
308 association between *n*MerTK and euchromatin on manually selected central nuclear  
309 regions (excluding the nucleoli) in the STORM images (Supplementary Fig. 5D). In  
310 addition, we estimated the degree of random colocalization occurring as a result of the  
311 high density of histones and *n*MerTK by generating *in-silico* images of randomly  
312 distributed *n*MerTK molecules (using the experimentally obtained *n*MerTK density in  
313 that particular cell). We super-imposed the *in silico* data to histone STORM images and

314 calculated the degree of random colocalization. A high degree of colocalization was  
315 found on the experimentally obtained STORM images, well-above random values and  
316 similar for day 0 DCs and for iDex cells (Fig. 5B). To further confirm that the observed  
317 colocalizations are real and not the result of cross-talk during imaging and/or cross-  
318 reactivity of the antibodies, we focused on areas where signal from only one of the two  
319 proteins is expected: cytosolic vesicles in the case of MerTK (Supplementary Fig. 5E)  
320 and the heterochromatin ring in the case of histones (Supplementary Fig. 5F). In both  
321 cases, the cross-talk was < 2%. These results thus confirm a high degree of spatial  
322 association between *n*MerTK and histones in central nuclear regions, strongly suggesting  
323 that *n*MerTK interacts with euchromatin in human DCs.

324

325 **Chromatin compaction increases upon DC differentiation and correlates with a  
326 reduction of *n*MerTK accumulation into nanoclusters**

327 Surprisingly, the results in Fig. 5B showed a similar degree of association between  
328 *n*MerTK and euchromatin regardless of DC differentiation state, i.e., day 0 and iDex,  
329 whereas the confocal data revealed higher levels of *n*MerTK in day 0 than in fully  
330 differentiated iDex cells (Fig. 2A). To understand this apparent discrepancy, we  
331 quantified the nanoscale organization of *n*MerTK on both cell types from STORM images  
332 using a cluster analysis algorithm as described by Ricci et al (Ricci et al., 2015) (see  
333 materials and methods). A much larger number of localizations per nanocluster was  
334 observed at day 0 compared to iDex (Fig. 5C) whereas the total number of nanoclusters  
335 per area was similar on both cell types (Supplementary Fig. 5G). These results thus reveal  
336 a direct correlation between *n*MerTK levels and nanocluster size, rather than nanocluster  
337 density. Altogether, these data suggest that *n*MerTK nanoclusters might constitute

338 functional units associated to chromatin that could more potently operate in newly  
339 differentiated DCs where *n*MerTK clusters are larger.

340 The results above naturally arise the question of how chromatin is organized on  
341 newly differentiated DCs (day 0) versus fully differentiated iDex (day 4). To address this  
342 question, we stained histone H2B in both cell types, performed STORM imaging over  
343 multiple cells (Fig. 5D) and compared their organization (see materials and methods).  
344 Interestingly, we found an increased number of H2B localizations per cluster (Fig. 5E) as  
345 well as increased cluster density (Fig. 5F) on fully differentiated iDex as compared to  
346 Day 0 DCs. The simultaneous increase in both parameters indicate that fully  
347 differentiated DCs have more histones covering their DNA and that chromatin is therefore  
348 more compact. We confirmed these results using conventional wide-field imaging,  
349 obtaining an increase of 30% in histone expression levels in the nucleus of iDex cells  
350 (Supplementary Fig. 6). These results are fully in line with a recent study showing a  
351 similar increase in the total histone content in pluripotent versus differentiated  
352 cells(Karnavas et al., 2014). An additional study also showed that histone content in  
353 monocytic-derived DCs can vary significantly upon treatment with different  
354 immunological stimuli (Parira et al., 2017). As H2B is directly involved in DNA  
355 compaction, our results reveal that chromatin is indeed in a more accessible conformation  
356 during early stages of DC differentiation, i.e., at day 0 DCs. Together, our observations  
357 that *n*MerTK shows increased clustering and preferentially interacts with chromatin  
358 exactly during this transcriptionally active stage where it is more accessible, together with  
359 its tendency to associate to euchromatin rather than to heterochromatin, strongly points  
360 towards a role for *n*MerTK as a genome regulator during DC differentiation.

361

362 ***n*MerTK has the potential to function as a transcription factor**

363 A few recent reports have speculated on the possible function for RTKs in the nucleus,  
364 and proposed roles in DNA replication (Wang et al., 2006), repair (Liccaldi et al., 2011)  
365 and/or transcription (Huo et al., 2010; Lin et al., 2001; Liu et al., 2010). Since DCs are  
366 non-proliferative cells, we conjectured that *n*MerTK could have a transcriptional  
367 function. Transcription factors are typically characterized by the presence of one or  
368 several transactivation domains that are involved in the recruitment of larger multiprotein  
369 complexes facilitating transcriptional activity (Raj and Attardi, 2017; Wärnmark et al.,  
370 2003). The sequence of these domains is highly conserved and can be predicted based on  
371 hydrophobicity and the presence of several key amino acids (Piskacek et al., 2016, 2007).  
372 We used an algorithm developed by Piskacek et al (Piskacek et al., 2007) to predict  
373 possible 9aaTAD (nine amino acid transactivation domain) regions in the MerTK  
374 sequence, and obtained two putative regions with a 100% match, within the cytoplasmic  
375 domain (Fig. 6A and Supplementary Fig. 7). This prompted us to test whether the  
376 cytoplasmic domain of MerTK indeed displays transactivational activity in a model cell  
377 system.

378 This intracellular domain, the extracellular domain and a positive control CREB  
379 (Sun et al., 1994), were fused to part of the DNA-binding protein GAL4, creating three  
380 different potential transcription factors (Fig. 6B). HeLa cells were co-transfected with a  
381 plasmid coding for one of these proteins, together with a reporter gene containing five  
382 GAL4 binding sites and a part coding for luciferase (Fig. 6B). The degree of luminescence  
383 found in the HeLa cells serves as a read-out for transcriptional activity induced by each  
384 of the possible transcription factors. As expected, the CREB fusion protein acted as a  
385 transcription factor and increased the transcription of the luciferase reporter gene  
386 compared to the control mock sample (Fig. 6C). The data was then normalized to the  
387 transcription induced by CREB, and displayed as a fold increase (Fig. 6C). Remarkably,

388 the MerTK-intracellular fusion protein showed an enormous potential as transcription  
389 factor, with an eight-fold increase in transcription as compared to CREB (Fig. 6C). The  
390 MerTK extracellular domain did not induce any transcription beyond that of the Mock  
391 control, fully in line with the prediction that only the intracellular domain contains  
392 9aaTAD sites and therefore transactivational capacity. Our data thus shows for the first  
393 time that MerTK has the potential to act as a potent transcription factor, strongly  
394 suggesting that *n*MerTK found in human DCs and associated to chromatin has the  
395 function to regulate gene transcription. Considering the time-sensitive dependence of  
396 nuclear accumulation of MerTK on DC differentiation, this transcription factor is likely  
397 to boost the upregulation of key genes during this process.

398

## 399 **Discussion**

400 In this study we have identified for the first time, to our knowledge, the presence of the  
401 transmembrane receptor MerTK in the nucleus of human DCs and show that the degree  
402 of nuclear localization strictly depends on DC maturation. Our super-resolution STORM  
403 studies on intact nuclei further revealed that *n*MerTK preferentially associates to open  
404 and active chromatin. We found that chromatin compaction increases upon DC  
405 differentiation and correlates with a reduction of *n*MerTK accumulation into  
406 nanoclusters. We also showed that MerTK has the potential to act as a powerful  
407 transcription factor, suggesting that this transmembrane receptor regulates the expression  
408 of key genes during DC differentiation.

409 Although MerTK has previously been observed in the nucleus of a leukaemia cell  
410 line (Jurkat T cells) (Migdall-Wilson et al., 2012), our study is the first to give functional  
411 importance to this intracellular localization in the context of immunity in primary human

412 cells. Based on our spatial, temporal and functional data, we suggest that *n*MerTK acts as  
413 a transcription factor involved in regulating the differentiation of human DCs in a time-  
414 sensitive manner. Interestingly, MerTK has been proposed to regulate the differentiation  
415 of natural killer (NK) cells by upregulating the membrane expression of certain key NK  
416 cell immunoreceptors during a well-defined period of cell maturation (Sun et al., 1994).  
417 The authors of that study envisioned that such upregulation happens through classical  
418 downstream signalling and activation of traditional transcription factors. However, they  
419 found that the upregulation was not caused by any of the known transcription factors  
420 involved in NK cell development. In the light of our work, it is highly conceivable that  
421 just like in DCs, MerTK translocates to the nucleus of NK cells to induce the upregulation  
422 of several important immunoreceptors, thereby regulating differentiation in a time-  
423 sensitive manner. A previous study by Schmahl et al. indeed suggested a similar  
424 regulating role for the FGF2a-RTK in the early stages of Sertoli cell differentiation  
425 (Schmahl, 2004). Further studies focusing on the genes that MerTK regulates as a  
426 transcription factor are necessary to fully understand how *n*MerTK directs DC  
427 differentiation. We persistently attempted to perform ChIP-Seq profiling experiments to  
428 identify genomic regions influenced by MerTK, as well as nuclear IP of MerTK followed  
429 by quantitative mass spectroscopy to detect the nuclear factors that MerTK forms a  
430 complex with. Unfortunately, these experiments turned out unfeasible due to the lack of  
431 validated MerTK antibodies for these techniques together with the enormous demand of  
432 cellular material that is incompatible with the isolation and culturing of monocyte derived  
433 DCs from blood.

434 Our super-resolution studies on tolerogenic DCs showed the presence of small  
435 MerTK nanoclusters on the cell membrane. Although receptor dimerization is expected  
436 to occur as a result of ligand activation by ProS in the medium, our observation of more

437 extensive nanoclustering is an important finding in the field of immunoreceptor  
438 organization at the cell surface. Membrane-bound MerTK expressed by tolerogenic DCs  
439 is thought to suppress the T cell response by depriving the local environment of ProS, a  
440 T cell activating factor (Cabezón et al., 2015; Carrera Silva et al., 2013). Efficient ProS  
441 scavenging from the local environment by MerTK requires rapid internalization of  
442 MerTK-ProS complexes in order to interfere with T cell binding and activation. We thus  
443 speculate that MerTK nanoclustering might provide an advantage for this rapid  
444 internalization by lowering the amount of energy and resources needed for ProS  
445 clearance. In addition, it is also conceivable that MerTK nanoclustering increases the  
446 binding affinity to ProS, favouring internalization. Moreover, the small number of  
447 MerTK molecules involved in each nanocluster (on average 3, ranging between 1 and 11)  
448 would provide an excellent strategy to optimize MerTK resources for efficient ligand  
449 scavenging throughout the local cell environment.

450 LRP-1 has been described as a receptor that regulates the protein composition of  
451 the plasma membrane (Gonias et al., 2004). In the context of immunity, LRP-1 regulates  
452 the membrane levels of  $\beta 1$  integrins (Theret et al., 2017; Wujak et al., 2018), CD44  
453 (Perrot et al., 2012) and the phagocytic receptor AXL (Subramanian et al., 2014) by  
454 facilitating their endocytosis. Knock-down or blocking of LRP-1 leads in all cases to an  
455 accumulation of the receptors at the membrane level. Our results on the role of LRP-1 in  
456 the partitioning and regulation of the spatial location of MerTK can be fully rationalized  
457 under the paradigm that LRP-1 controls the composition of the cell membrane. Like the  
458 previously mentioned receptors, we found that MerTK accumulates at the cell membrane  
459 when LRP-1 expression levels are low, which physiologically occurs during the final  
460 stages of DC differentiation (day 4). However, while LRP-1 targets many other receptors  
461 towards lysosomal degradation or recycling, we show here that this receptor is also

462 involved in nuclear translocation. It was shown previously that LRP-1 can target soluble  
463 toxins from the cell environment into the nucleus in a receptor-ligand fashion (Chaumet  
464 et al., 2015), but to our knowledge LRP-1 has never been implicated in chaperoning other  
465 transmembrane proteins towards the nucleus. We thus propose that the subcellular spatial  
466 destination of MerTK is tuned by LRP-1. When DCs simultaneously express both MerTK  
467 and LRP-1, LRP-1 will bind MerTK and will direct it into the nucleus (day 0) via  
468 endocytosis. By contrast, in the absence of, or at reduced LRP-1 levels, assistance for  
469 nuclear translocation is compromised, and MerTK remains at the surface (day 4).  
470 Pinpointing, for the first time, the role of LRP-1 in this process is a great step forward in  
471 the molecular understanding of nuclear trafficking of transmembrane receptors. This  
472 knowledge can be used to further our understanding of nuclear translocation of other  
473 RTKs. Since many other RTKs play an oncogenic role in the nucleus, identifying triggers  
474 in this process is of paramount importance for future clinical interference.

475 The presence of full-length RTKs in the nucleus has been reported for a significant  
476 number of receptors over the last decade (reviewed in (Brand et al., 2012; Wang and  
477 Hung, 2012; Wells and Marti, 2002a)). However, the community has been rather reluctant  
478 to accept these observations, partially because it is counter-intuitive to envision how full-  
479 length membrane-bound receptors containing a hydrophobic transmembrane domain  
480 could be soluble inside the nucleoplasm. The existence of RTKs with a deleted  
481 transmembrane domain has been proposed in a model in which those mutated proteins  
482 dimerize with their wild-type counterparts (Wells and Marti, 2002a). The dimerization  
483 would provide ligand sensitivity and explain its localization at the cell membrane as well  
484 as the membrane of intracellular compartments. Such a soluble, almost full-length  
485 isoform has been detected for the FGFR2 receptor (Katoh et al., 1992), but for many other  
486 RTKs in the nucleus, including MerTK, the existence of such an isoform has never been

487 demonstrated. Moreover, the specific presence of the transmembrane domain in the  
488 nucleus has been shown in some cases (Wells and Marti, 2002b), contradicting this  
489 model. Alternatively, we hypothesize that although not incorporated in the lipid bilayer,  
490 the transmembrane domain could still be covered by a small amount of lipids in the  
491 nucleus. The concept of nuclear lipids has been widely described over the past two  
492 decades, and the existence of proteolipid complexes has been observed (Albi and Magni,  
493 2004; Cascianelli et al., 2008; Irvine, 2000). *In vitro* experiments along these lines will  
494 be important to further understand the intriguing phenomenon of soluble transmembrane  
495 receptors as nuclear regulators.

496 A second aspect discouraging the investigation of nuclear localization of  
497 membrane proteins is the general consensus that aberrant or overexpression of the protein  
498 causes this atypical nuclear translocation, and that the presence of RTKs in the nucleus is  
499 mostly related to malignancies. Our results on MerTK are however markedly distinct in  
500 several ways: *First*, *n*MerTK is found in a very high concentration in healthy non-  
501 proliferating DCs. *Second*, *n*MerTK localization seems to be exclusively reserved for  
502 immune cells (directly isolated DCs, monocyte-derived DCs and THP-1 cells in our  
503 experiments, and Jurkat T cells (Migdall-Wilson et al., 2012)). Indeed, we showed that  
504 overexpression of MerTK in other cell types, both with (HEK293) and without (HeLa)  
505 endogenous MerTK, does not lead to nuclear accumulation of the receptor. *Third*, the  
506 degree of nuclear translocation strictly relates to DC differentiation, a physiological  
507 process that is crucial to immunity. The sharp peak of *n*MerTK accumulation we observed  
508 in newly differentiated DCs suggests a critical time-sensitive and well-regulated need for  
509 the presence of the receptor in the nucleus during differentiation. Our results thus  
510 interestingly suggest a physiological, non-malignant role for a RTK in the nucleus,

511 validating the importance of further studies on this puzzling and unconventional way of  
512 cell signalling.

## 513 Materials and methods

514 **Cell culture.** Dendritic cells were derived, as reported previously(de Vries et al., 2005),  
515 from peripheral blood samples. Buffy coats from healthy donors were obtained  
516 from *Banc de Sang i Teixits* upon written informed consent. In brief, peripheral blood  
517 mononuclear cells (PBMCs) were allowed to adhere to a plastic surface for 2 h at 37°C.  
518 Unbound PBMCs were washed away, and the remaining adherent monocytes were  
519 cultured for 48h in the presence of IL-4 (300 U/ml) and GM-CSF (450 U/ml) (both from  
520 Miltenyi Biotec, Madrid, Spain) in X-VIVO 15 (BioWhittaker, Lonza Belgium) medium  
521 supplemented with 2% AB human serum (Sigma-Aldrich, Spain). At that moment, they  
522 are day 0 DCs, and were used for several experiments. To generate iDexs, the cells were  
523 further cultured for 4 days in the same conditions plus Dexamethasone (1  $\mu$ M; MERCK).  
524 IDCs were equally generated in 4 days, but without the extra addition of Dex. For serum  
525 starvation experiments, monocytes were cultured normally up to day 0. Then, they were  
526 differentiated into iDex DCs using cytokines and Dex, but using a lower concentration of  
527 HS (1% instead of 10%). This percentage was experimentally determined as the lowest  
528 concentration at which the DCs still developed normally (assessed visually). During the  
529 last 48h of differentiation, recombinant human ProS was added to one of the conditions  
530 (concentration according to Cabezón et al. (Cabezón et al., 2015)).  
531 THP-1 cells were cultured in RPMI 1640 medium supplemented with antibiotic-  
532 antimycotic (both Gibco) and 10% FBS (ThermoFisher). To induce a DC-like phenotype,  
533 they were cultured for 6 days in the presence of IL-4 and GM-CSF, with a medium  
534 exchange after 3 days.  
535 HeLa cells and Hek293 cells were both cultured in complete medium (Dulbecco's  
536 modified Eagle's medium containing 10% fetal bovine serum (both Gibco)).

537 **Antibodies and reagents.** The following primary antibodies were used throughout this  
538 study at a concentration of 5 µg/ml, except for the STORM experiments where they were  
539 used at a concentration of 20 µg/ml:  $\alpha$ -MerTK (mouse extracellular monoclonal, 125618,  
540 R&D Systems),  $\alpha$ -MerTK (goat extracellular polyclonal, AF891, R&D Systems),  $\alpha$ -  
541 MerTK (rabbit extracellular monoclonal, Y323, Abcam),  $\alpha$ -MerTK (rabbit intracellular  
542 polyclonal phosphospecific, PMKT-14GAP, FabGennix),  $\alpha$ -ProS-AF647 (bs-9512R-  
543 A647, Bioss),  $\alpha$ -LAMP1 (H5G11, Santa Cruz Biotechnology),  $\alpha$ -calreticulin (ADI-SPA-  
544 601, Enzo),  $\alpha$ -EEA1 (14/EEA1, BD Biosciences),  $\alpha$ -LRP-1 (LRP1-11, Sigma-Aldrich),  
545  $\alpha$ -PanHis (H11-4, Merck Millipore),  $\alpha$ -H2B (5HH2-2A8, Merck Millipore),  $\alpha$ -HDAC1  
546 (10E2, Cell Signalling),  $\alpha$ -tubulin (YL1/2, Abcam).  
547 For confocal and STED imaging, the following secondary antibodies were used, all at a  
548 concentration of 10ug/ml: Goat- $\alpha$ -mouse-AF488 (A11001, ThermoFisher), Goat- $\alpha$ -  
549 mouse-Atto647N (50185, Sigma-Aldrich), Goat- $\alpha$ -rabbit-AF488 (A11008,  
550 ThermoFisher), Goat- $\alpha$ -rabbit-AF647 (A21244, ThermoFisher).  
551 For Western blot imaging, the following secondary antibodies were used (all from  
552 ThermoFisher): Donkey- $\alpha$ -rabbit-AF680 for MerTK, Donkey- $\alpha$ -mouse-AF680 for  
553 HDAC1 Donkey- $\alpha$ -rat-DyLight800 for tubulin.  
554 For STORM imaging, the secondary antibodies (donkey- $\alpha$ -mouse and donkey- $\alpha$ -rabbit  
555 from ImmunoResearch, used at a concentration of 20 µg/ml) were labelled in-house with  
556 different combinations of pairs of activator/ reporter dyes. The dyes were purchased as  
557 NHS ester derivatives: Alexa Fluor 405 Carboxylic Acid Succinimidyl Ester (Invitrogen),  
558 Cy3 mono-Reactive Dye Pack (GE HealthCare), and Alexa Fluor 647 Carboxylic Acid  
559 succinimidyl Ester (Invitrogen). Antibody labelling reactions were performed by  
560 incubating a mixture of secondary antibody, NaHCO<sub>3</sub>, and the appropriate pair of  
561 activator/reporter dyes diluted in DMSO for 40 min at RT. Purification of labelled  
562 antibodies was performed using NAP5 Columns (GE HealthCare). The dye to antibody  
563 ratio was quantified using Nanodrop and only antibodies with a composition of 3-4 Alexa  
564 Fluor 405 and 0.9-1.2 Alexa Fluor 647 per antibody were used for imaging.

565 Recombinant human PROS1 (R&D systems, 50 nM final concentration) and recombinant  
566 human RAP (Merck Millipore, 200 nM final concentration) were used to stimulate  
567 nuclear translocation of MerTK.

568

569 **Flow cytometry.** For flow cytometry analysis, DCs were labelled with primary antibody  
570  $\alpha$ -MerTK (R&D systems), followed by secondary staining with PE-labelled goat-anti-  
571 mouse (from BD Biosciences), both for 30 min at 4°C and a concentration of 5  $\mu$ g/ml.  
572 Appropriate isotype control IgG1 (from BD Biosciences), was included. Flow cytometry  
573 was performed using FACSCanto II.

574

575 **Plasmids.** The MerTK (Mer cDNA ORF Clone, Human, untagged, pCMV3) was  
576 obtained from Sino Biological. Both pcDNAI-GAL4-CREB and 5xGAL4-TATA-  
577 luciferase were a gift from Richard Maurer(Sun et al., 1994) (Addgene plasmid # 46769  
578 and Addgene plasmid # 46756, respectively). For transfection experiments of MerTK in  
579 different cancer cell lines, a GFP-tag was added to the plasmid. For the luciferase assay,  
580 we cloned the pcDNAI-GAL4-MerTK-extracellular and the pcDNAI-GAL4-MerTK-  
581 intracellular constructs by Gibson assembly of two fragments, the first one obtained by  
582 digesting the pcDNAI-GAL4-CREB vector with EcoRI and XbaI restriction enzymes  
583 (New England Biolabs), and the second part obtained by amplifying either the  
584 extracellular or the intracellular coding regions of the MerTK vector by PCR. The primers  
585 used for these amplifications (obtained from Integrated DNA technologies) were 5'-  
586 AGTAGTAACAAAGGTCAAAGACAGTTGACTGTATGCCCGAATTGCTATC  
587 ACTGAGGCAAGGGAAAGAAG-3' and 5'GATCCTCTAGCATTAGGTGACACTA  
588 TAGAATAGGGCCCTCTAGAGATGATGAGCACAGGATCTTAGTT-3' for the  
589 extracellular domain of MerTK (residues 21–505) and 5'-AGTAGTAA  
590 CAAAGGTCAAAGACAGTTGACTGTATGCCCGAATTCAAAGAGTCCAGGA  
591 GACAAAGTTGG-3' and 5'-GATCCTCTAGCATTAGGTGACACTATAGAAT  
592 AGGGCCCTCTAGATTACATCAGGACTTCTGAGCCTCTGAGGAGT-3' for the  
593 cytosolic domain of MerTK (residues 527–999). SnapGene software (obtained from GSL  
594 Biotech) was used for molecular cloning procedures.

595 **MerTK transfection.** HeLa cells were transfected using TransIT-HeLaMONSTER and  
596 HEK293 cells using TransIT-293 (both from Mirus). Cells after transfection were  
597 cultured both with and without ProS or HS in the medium, to potentiate nuclear  
598 translocation of MerTK. Cells were typically imaged 24h after transfection, although both  
599 earlier and later time points were also explored.

600

601 **Transcriptional activation luciferase assay.** HeLa cells were seeded on a 24-well plate,  
602  $2.5 \times 10^4$  cells per well. After 24 hours, cells were cotransfected with both the reporter  
603 gene and one of the different putative transcription factors using X-tremeGENE 9 (Roche)  
604 following the manufacturer's recommendations. The cells received 1 unit of 5xGAL4-  
605 TATA-luciferase reporter DNA and 0.4 units of the putative transcription factor DNA.  
606 48 hours after transfection, cells were lysed with 100  $\mu$ l of cell culture lysis reagent  
607 (Promega, Luciferase Assay System Kit #E1500) for 10 min on ice, and then spun down  
608 at 12000 g for 2 min at 4°C. For the luciferase assay we mixed 20  $\mu$ l of those supernatants  
609 with 100  $\mu$ l of the luciferase assay reagent (Promega, Luciferase Assay System Kit  
610 #E1500) in a well of a white, opaque 96-well plate, and the luminescence was measured  
611 after 30 sec using a manual luminometer (Gen5 microplate reader, BioTek), programmed  
612 to perform a 10 sec measurement read for luciferase activity. Luminescence was  
613 normalized and represented as the fold increase relative to the luminescence induced by  
614 positive control fusion protein GAL4-CREB. Each transfection was performed in  
615 triplicates, and the experiments were repeated 3 times in different days.

616

617 **Cell fractionation and Western blot detection.** Dendritic cells at day 0 and DC-like  
618 THP-1 cells were collected and fractionated into the cytoplasmic fraction, the soluble  
619 nuclear fraction and the chromatin bound fraction following Wang et al.(Wang et al.,  
620 2015). In brief, cells were crushed using a Dounce tissue grinder set (Sigma-Aldrich) of  
621 2ml, which homogenizes the cells without rupturing the nuclear membrane. Effectivity  
622 of this step was checked under the microscope with a Trypan Blue staining. Cytoplasmic  
623 material was then separated from the intact nuclei by centrifugation. The nuclei were  
624 subsequently lysed, and the chromatin was separated from the soluble fraction by

625 centrifugation. The chromatin pellet was then sonicated in order to release associated  
626 proteins and allow their detection. All 3 fractions were then loaded and ran, transferred,  
627 and stained following standard Western blotting procedure. Besides MerTK, we also  
628 stained for tubulin and HDAC1 to verify the effectiveness of the cell fractionation.

629

630 **Sample preparation for fluorescence imaging.** Fresh cells were diluted up to a  
631 concentration of  $1 \times 10^6$  per ml in plain medium, and attached to the bottom of the cover  
632 glasses (Lab-Tek) by incubation for 30 min. Samples were then fixed using 4%  
633 paraformaldehyde (PFA) for 15 min at RT. Then, cells were blocked and permeabilized  
634 for 1h at RT with 3% BSA and 0,5% TritonX-100 in PBS, followed by primary and  
635 secondary labelling both for 30 min at RT. Finally, all samples were fixed again with 2%  
636 PFA and stored at 4 °C. For membrane staining, TritonX-100 was left out from the  
637 blocking mixture.

638

639 **Confocal imaging.** Imaging was performed using a confocal microscope (TCS SP5,  
640 Leica Microsystems). Images were taken with a 1.4 NA oil immersion objective (HCX  
641 PL APO CS 63.0x, Leica), a  $512 \times 512$  pixels format and a scanning speed of 400 Hz.  
642 AF488 was excited with the 488 nm line, at 25% of the argon laser power and detected  
643 between 500 nm and 570 nm. Atto647N or AF647 was excited with the 633 nm line at  
644 30% of the HeNe laser power and detected between 645 nm and 715 nm. To be able to  
645 use the fluorescence intensity measurements in a quantitative way, imaging conditions  
646 were always kept constant across measurements, and a calibration sample was used to  
647 account for day to day fluctuations in the system.

648

649 **STED imaging.** Imaging was performed using a commercial STED microscope (TCS  
650 SP5, Leica Microsystems). Images were taken with a 1.4 NA oil immersion objective  
651 (HCX PL APO CS 63.0x, Leica), a  $1024 \times 1024$  pixels format and a scanning speed of  
652 1400 Hz. The effective imaging beam consisted of the 488 nm line, at 25% of the argon  
653 laser power, and 100% of the depletion donut-shaped laser at 592 nm. Fluorescence was  
654 collected between 500 nm and 570 nm.

655 **STORM imaging.** Imaging was performed using a commercial microscope system from  
656 Nikon Instruments (NSTORM). Samples were prepared as described above, and imaged  
657 in the following buffer to facilitate blinking: Cysteamine MEA (Sigma-Aldrich), Glox  
658 Solution (0.5 mg/ml glucose oxidase, 40 µg/ml catalase; both Sigma Aldrich) and 10%  
659 Glucose in PBS(Bates et al., 2007). Images were acquired with a frame rate of 83 frames  
660 per second. In single color experiments, H2B was stained with the AF405-AF647  
661 activator/reporter dye pair. By exciting AF405 with the corresponding laser line at 405  
662 nm, this dye becomes activated and transfers its photons to the reporter dye. The reporter  
663 dye in turn will emit these photons only upon excitation with a 647 nm laser, and  
664 subsequently goes back into the dark state. We therefore used an imaging cycle in which  
665 one frame belonging to the activating light pulse (405 nm) was alternated with 3 frames  
666 belonging to the imaging light pulse (647 nm). Dual colour imaging was performed with  
667 two sets of secondary antibodies labelled with the same reporter dye (Alexa Fluor 647)  
668 but two different activator dyes (Alexa Fluor 405 for MerTK and Cy3 for panHis)(Bates  
669 et al., 2007). In addition to the first imaging cycle of 4 frames, a second cycle of 4 frames  
670 with an activation laser pulse at 561 nm was used to image Alexa Fluor 647 linked to  
671 activator Cy3.  
672 In order to exhaustively image all fluorophores in a reproducible manner allowing for  
673 quantitative comparison across cells and conditions, we used the following scheme to  
674 increase the activator laser power, according to Ricci et al. (Ricci et al., 2015).  
675

Activating laser power	frames
8 mW	Until 20.000
9 mW	20.000 - 37.000
10 mW	37.000 - 50.000
11 mW	50.000 - 57.000
13 mW	57.000 - 63.000
15 mW	63.000 - 70.000
18 mW	70.000 - 75.000

22 mW	75.000 - 80.000
29 mW	80.000 - 85.000
38 mW	85.000 - 90.000
48 mW	90.000 - 95.000
60 mW	95.000 - 100.000

676

677 **STORM image reconstruction.** STORM images were processed and rendered as  
678 previously described (Bates et al., 2007). Briefly, spots in single-molecule images were  
679 identified based on a threshold and fit to a Gaussian to identify their position in x and y.  
680 Applying this approach over all 100.000 frames gives the raw STORM data, consisting  
681 of a list of x-y coordinates, corresponding to the localized positions of all the  
682 fluorophores. Reconstructed images from the x-y coordinates were displayed using  
683 Insight3, after both drift and crosstalk correction following Refs 30 and 31.

684 Grouping of the x-y localization into clusters was done according to Ricci et al. (Ricci et  
685 al., 2015) using a custom-made cluster analysis algorithm written in MatLab. First, a  
686 density map was generated, in which each pixel has a value equal to the number of  
687 localizations falling within the pixel area (pixel size = 10 nm). A constant threshold was  
688 then used to convert the density maps into binary images, such that pixels have a value  
689 of 1 where the density is larger than the threshold and a value of 0 elsewhere.  
690 Localizations falling on zero-valued pixels were discarded from further analysis. For our  
691 threshold setting, the number of discarded localizations typically corresponded to < 5%  
692 of the total number of localization within a nuclear region. Connected components of the  
693 binary image, composed by adjacent non-zero pixels (4-connected neighbours), were  
694 sequentially singled out and analysed. Localization coordinates within each connected  
695 component were grouped by means of a distance-based clustering algorithm.  
696 Initialization values for the number of clusters and the relative centroid coordinates were  
697 obtained from local maxima of the density map within the connected region, calculated  
698 by means of a peak finding routine. Localizations were associated to clusters based on  
699 their proximity to cluster centroids. New cluster centroid coordinates were iteratively  
700 calculated as the average of localization coordinates belonging to the same cluster. The

701 procedure was iterated until convergence of the sum of the squared distances between  
702 localizations and the associated cluster and provided cluster centroid positions and  
703 number of localizations per cluster.

704

705 **Image analysis.** All image analysis was performed using ImageJ unless otherwise stated.  
706 Nuclear MFI (mean fluorescence intensity) from confocal images was quantified by  
707 manually selecting the nuclear area based on the transmission images of the cells.  
708 Colocalization was determined using the plugin Coloc2, and quantified by using the  
709 Pearson correlation coefficient for raw images, or the Mander's overlap coefficient for  
710 binary images (in the case of the dual colour STORM images). Image segmentation was  
711 performed according to Rizk et al (Rizk et al., 2014), using their plugin. STED images  
712 were analysed using a custom-written routine in MatLab. From these images, the number  
713 of MerTK molecules per nanocluster on the membrane was estimated by dividing the  
714 background-corrected fluorescence intensity of each MerTK spot by the average intensity  
715 of the spots on the glass (single labelling units of 1 primary and several secondary  
716 antibodies). The physical size of the clusters was calculated by taking the FWHM (full  
717 width half max) of the fitting of the fluorescence intensity profile of each spot. The size  
718 of the spots on the glass provide the effective resolution of our STED system, around 100  
719 nm.

720

721 **Statistical Analysis.** All analyses were performed using GraphPad Prism 6. Results are  
722 shown as the mean  $\pm$  SD. To determine statistical differences between the mean of two  
723 data sets, the (un)paired two-tailed Student T-test was used. To determine statistical  
724 differences between the mean of 3 or more data sets, the One-way ANOVA was used,  
725 followed by the Tukey's multiple comparison test. On single-cell data coming from  
726 different donors, an average value per donor was used to calculate statistical differences.  
727 Significance is represented using: ns (P>0.05); \* (P<0.05); \*\* (P<0.001) and \*\*\*  
728 (P<0.0001).

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739

740 **Competing interests:**

741 The authors declare non-financial competing interests.

742

743 **Author contributions:**

744 K.J.E.B., F.C., M.F.G.-P. designed the research. K.J.E.B., G.F.-G., and F.C. performed  
745 the experiments. K.J.E.B. and M.A.R performed STORM imaging. C.M. developed  
746 STORM analysis algorithms and provided advice on the analysis. M.L., A.C., D.B.-R.,  
747 discussed data and hypotheses. K.J.E.B. and M.F.G.-P. wrote the manuscript. All the  
748 authors reviewed the manuscript and provided feedback.

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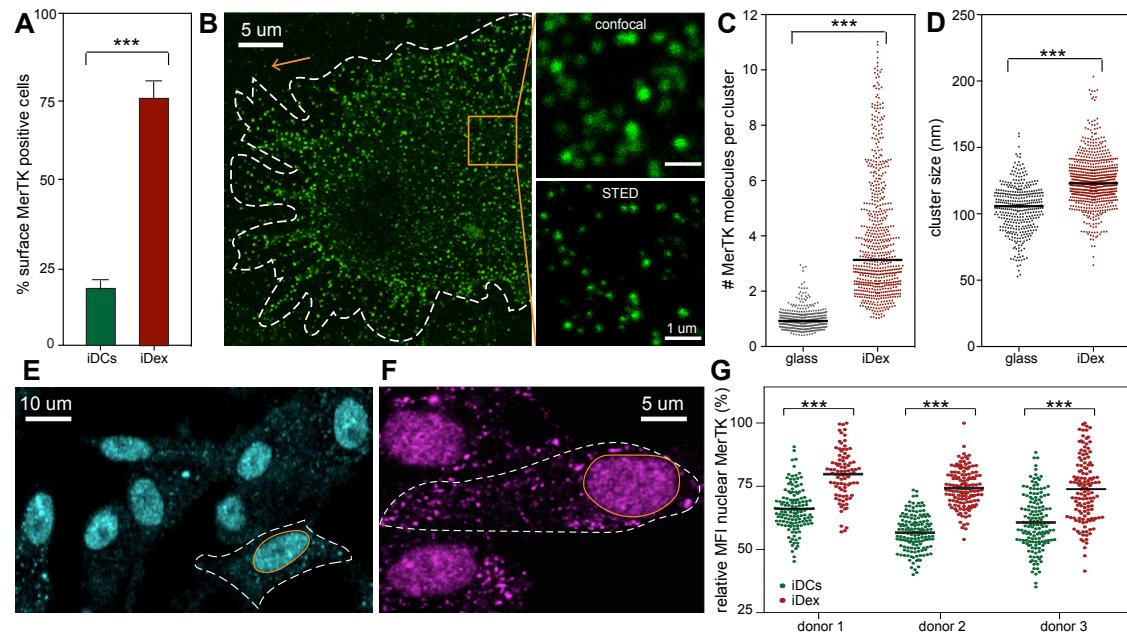
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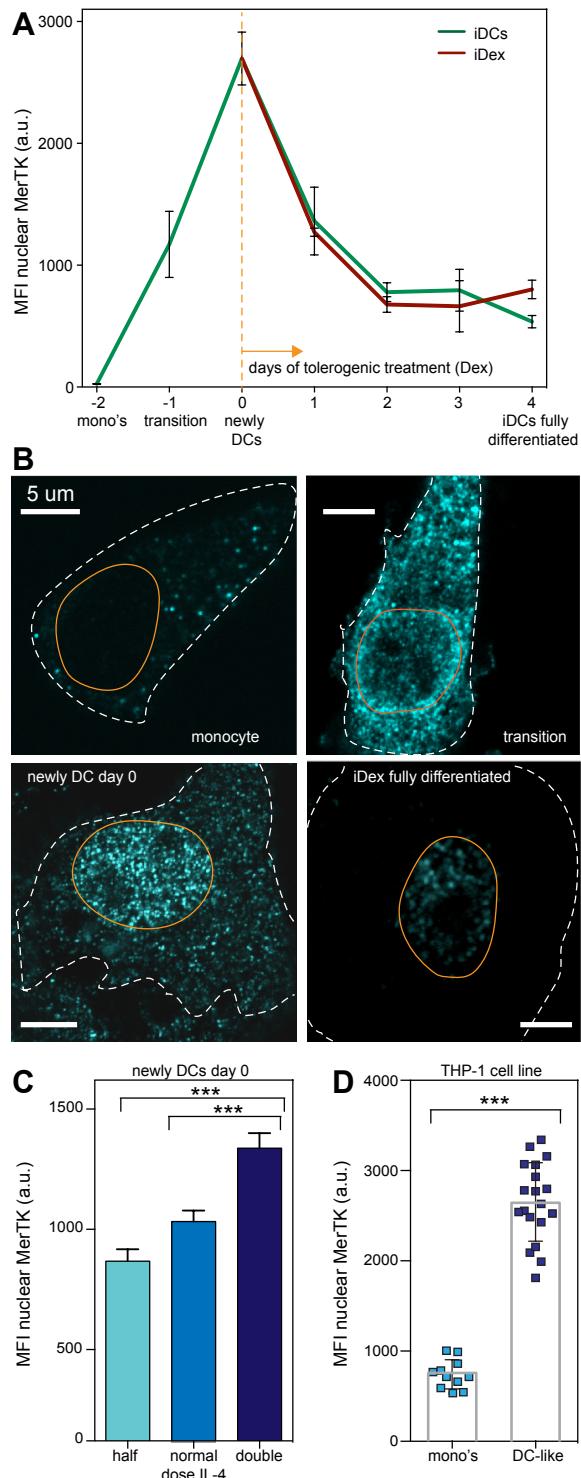
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## Figures

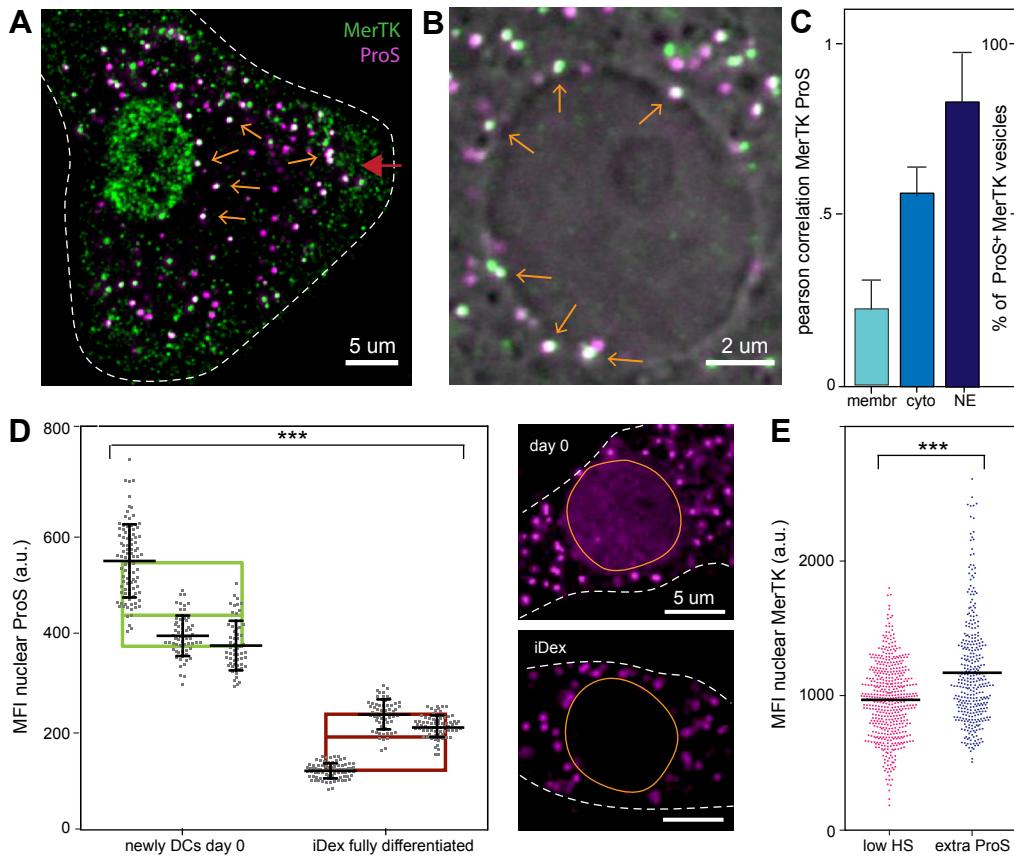


**Figure 1: Membrane and intracellular distribution of MerTK in DCs.** (A) Flow cytometry analysis of surface MerTK expression on iDC and iDex cells (n=8). (B) Representative STED image of MerTK on the plasma membrane of an iDex cell. The dotted line delineates the cell boundary. The orange square indicates the location of the zoom-in images, shown in confocal and STED modes. The orange arrow points to an individual 'labelling unit' on the glass that is used for the quantification in C. (C) Quantification of the number of MerTK molecules per nanocluster in iDexs, compared to the intensity of individual labelled antibodies on glass. (D) MerTK nanocluster sizes. Data from 3 different donors (around 8 cells each) for C and D. (E) Representative confocal image of the intracellular distribution of MerTK, using a MerTK Ab against an extracellular epitope. The dotted line represents the cell boundary, while the orange line represents the nuclear envelope. (F) Like in E, but using a MerTK Ab against an intracellular epitope. (G) Relative mean fluorescence intensity (MFI) of MerTK in the nucleus of iDCs and iDexs. Each dot corresponds to a single nucleus: Data from 80-100 cells per donor per condition.

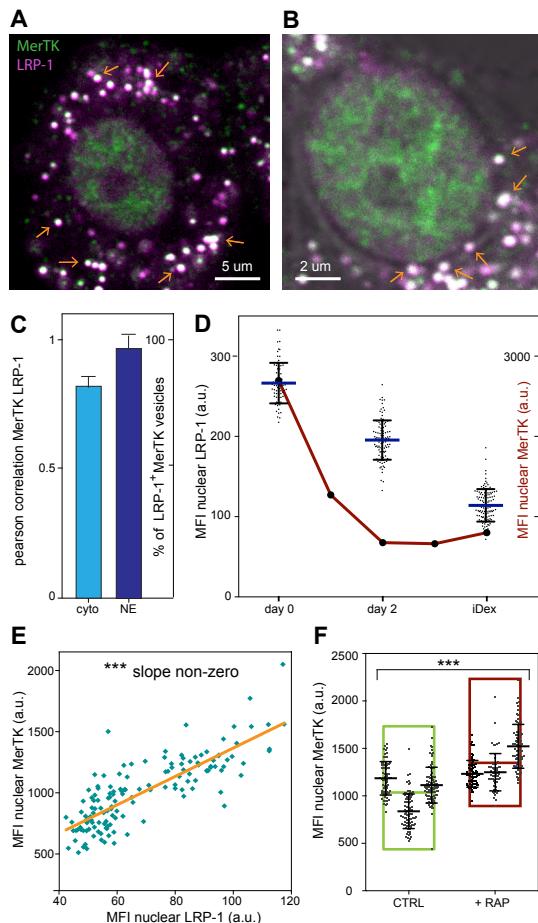


**Figure 2: Nuclear expression of MerTK during monocyte differentiation into DCs.**

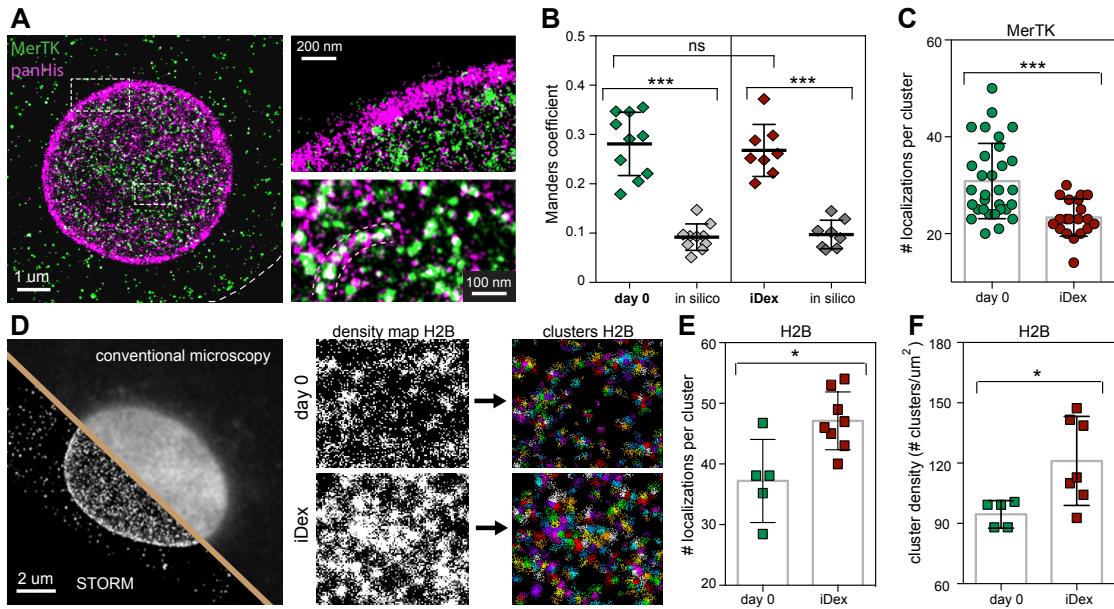
**(A)** MFI of *n*MerTK at different time points during monocyte differentiation into DCs. Day -2 (mono's) corresponds to harvested monocytes, 3 hours after removal of all other leukocytes. Day -1 (transition) corresponds to 24 hours after inducing differentiation. Day 0 (newly DCs) corresponds to 48 hours after inducing differentiation. At day 0, one pool of cells were subjected to Dex treatment (iDex) and the other pool left without the treatment. For both pools, *n*MerTK was measured until fully differentiated DCs were obtained (day 4). 25-50 cells from 3 different donors per condition. **(B)** Representative confocal images of intracellular MerTK distribution in differentiating monocytes at different time points. Of note, the loss of MerTK signal at the plasma membrane is mostly due to the robust permeabilization treatment required to penetrate the nucleus. **(C)** MFI of *n*MerTK as a function of IL-4 dose during the first 2 days of differentiation (day -2 to day 0). Around 80 cells from 2 different donors per condition. **(D)** MFI of *n*MerTK in THP-1 cells before and after differentiation towards a DC-like phenotype. N=20.



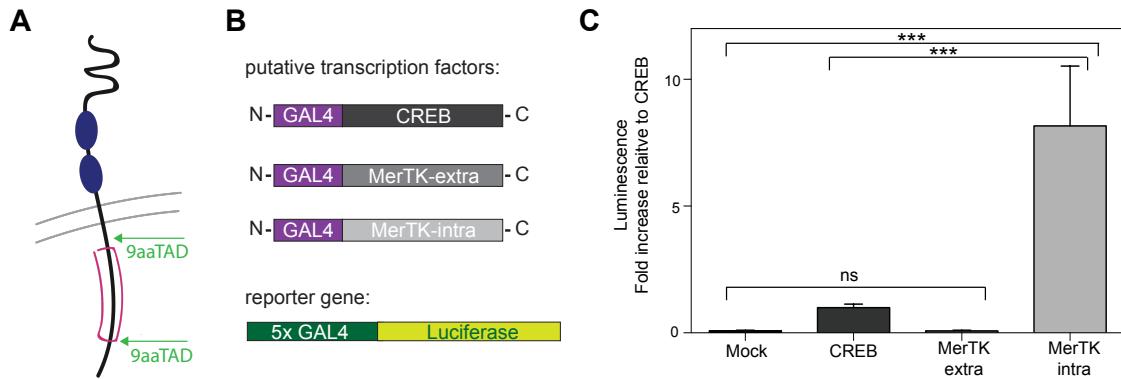
**Figure 3: Effect of ligand ProS binding on translocation of *n*MerTK. (A)** Representative dual colour confocal image of MerTK and its ligand ProS at day 0. MerTK is shown in green, ProS in magenta. Orange arrows indicate white spots in which both molecules clearly colocalize. The red arrow indicates the flattest part of the cell where the apical membrane is in focus. **(B)** Zoomed in on the nuclear area and overlaid with a transmission image to indicate the nucleus and its surrounding envelope. Orange arrows indicate spots of MerTK-ProS colocalization that are associated to the NE. Cells are minimally permeabilized in order to clearly observe the fluorescent spots at the NE. In these conditions there is minimal penetration of the antibodies into the nucleus. **(C)** Quantification of colocalization as a function of the cell region, i.e., membrane, cytoplasm and NE. Areas with the apical membrane in focus were chosen for the membrane portion of the analysis, the rest of the cell body excluding the nucleus was categorized as the cytoplasm. On zoom-in images like B, we manually counted the percentage of MerTK spots at the NE that colocalize with ProS. 10-20 cells from 2 different donors. **(D)** MFI of *n*ProS at day 0 and after full differentiation into iDexs. Each spot represents a single nucleus; the smaller plots provide representative fluorescence images of ProS at these time points. **(E)** MFI of *n*MerTK with and without the addition of extra recombinant human ProS during culture. Data from 3 different donors.



**Figure 4: Role of LRP-1 in translocation of *n*MerTK. (A)** Representative dual colour confocal image of MerTK (green) and LRP-1 (magenta) intracellularly. Orange arrows point to spots of clear colocalization between both

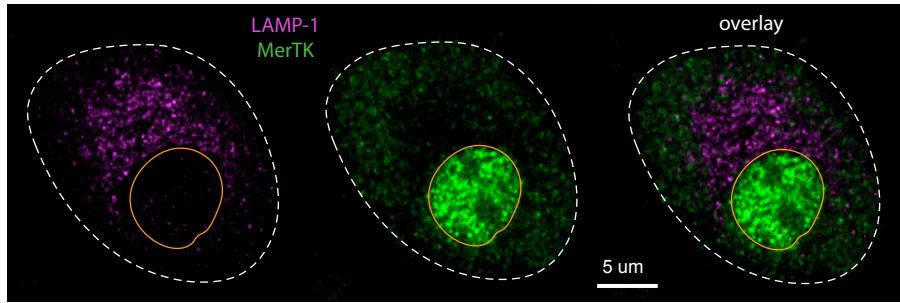


**Figure 5: Dual colour super resolution STORM imaging of nMerTK and chromatin. (A)** Representative reconstructed dual colour STORM image of nMerTK (green) and panHis (magenta) in the nucleus of a day 0 DC. The nucleus is delineated by a dense ring of histones (heterochromatin). The dotted white line indicates the cell boundary. The panels on the right correspond to zoom in images at two different nuclear areas. Elongated structures composed of nMerTK and histones are visible in the bottom zoom-in image (dotted pink lines). **(B)** Quantification of the colocalization between panHis and nMerTK (after image processing shown in Suppl. Fig. 5D) using the Manders overlap coefficient. The degree of colocalization was determined at day 0 and iDex, and was in both cases compared to the degree of colocalization between the experimental distribution of panHis and a random distribution of MerTK (*in silico*). Each symbol in the plot corresponds to an individual nucleus analysed. Data from 2 different donors. **(C)** Quantification of the number of localizations per nMerTK nanocluster in the nucleus of both day 0 and iDex DCs. Each dot corresponds an individual cell, with an averaged value from hundreds of nanoclusters per cell. Data from 3 different donors. **(D)** Representative single-color reconstructed STORM image of H2B in the nucleus of a day 0 DC. The image was partially overlaid with a conventional image of H2B in the same cell, to show the increase in resolution gained by using STORM. The panels on the right show the image processing strategy to generate density maps of the H2B signal (see methods for details). **(E)** Quantification of the number of localizations per H2B nanocluster in day 0 and iDex DCs. **(F)** Quantification of the H2B nanocluster density (the number of nanoclusters per  $\mu\text{m}^2$ ) in day 0 and iDex DCs. Each square in (E) and (F) corresponds to the average value over hundreds of H2B nanoclusters/cell of a single cell.

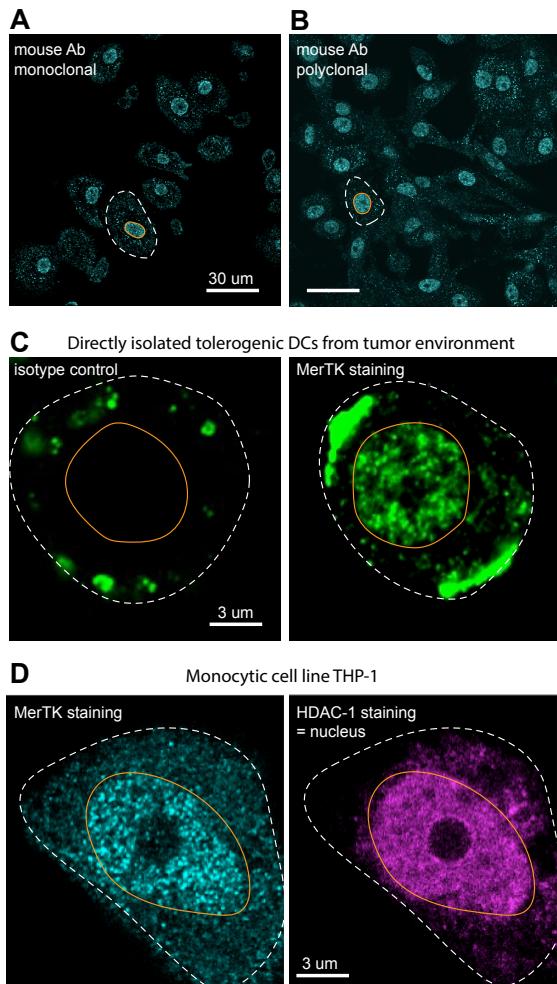


**Figure 6: Transactivational activity of MerTK.** (A) Schematic representation of MerTK topology (extracellular up) with predicted sites of 9aaTAD transactivation domains (both intracellular). (B) Schematic representation of the 3 fusion proteins that serve as putative transcription factors and the corresponding reporter gene. HeLa cells were co-transfected with one of the transcription factors and the reporter gene. (C) Quantification of the transcription induced by the different putative transcription factors, calculating the amount of luminescence generated after each co-transfection as described in (B), or Mock transfection as a negative control. Experiments were performed in triplicate and repeated 3 times during different cell line passages.

## Supplementary Figures

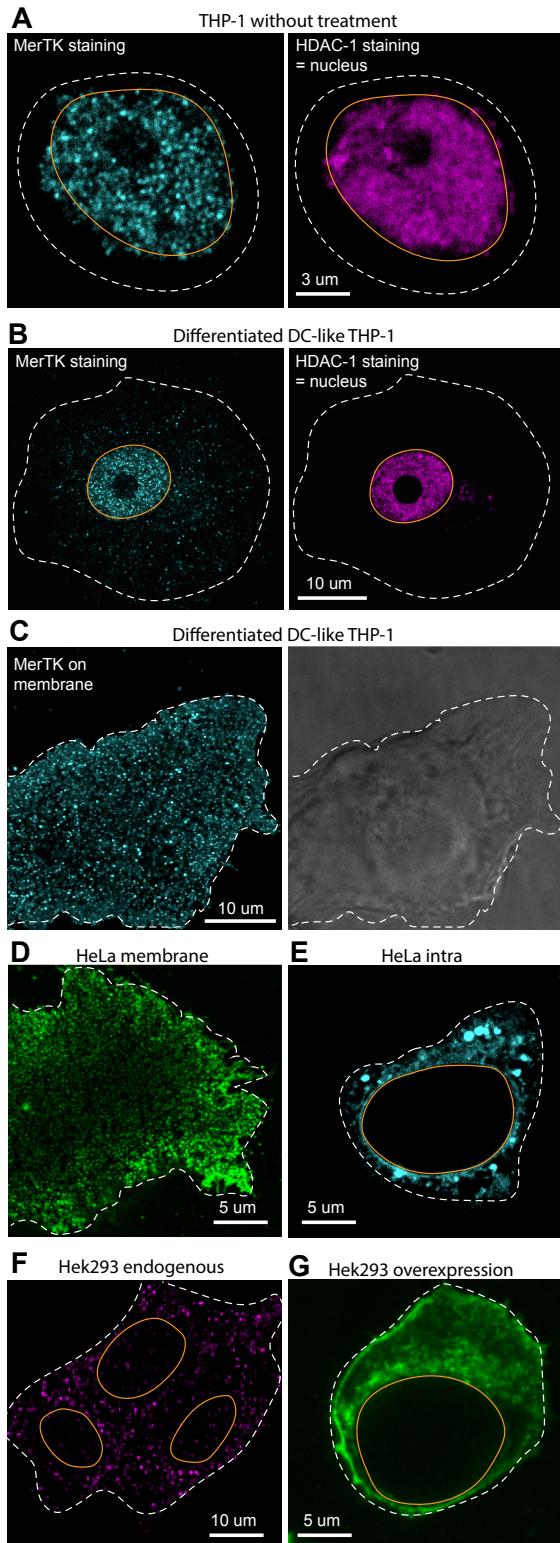


**Figure S1: Intracellular MerTK does not reside in lysosomes.** Representative dual colour confocal image of LAMP-1 staining the lysosomes in magenta, and MerTK in green. There is clear antilocalization visible between both components. The dotted line indicates the cell boundary, and the orange line the nuclear envelope.



**Figure S2: Nuclear staining of MerTK in different monocyte immune cells.**

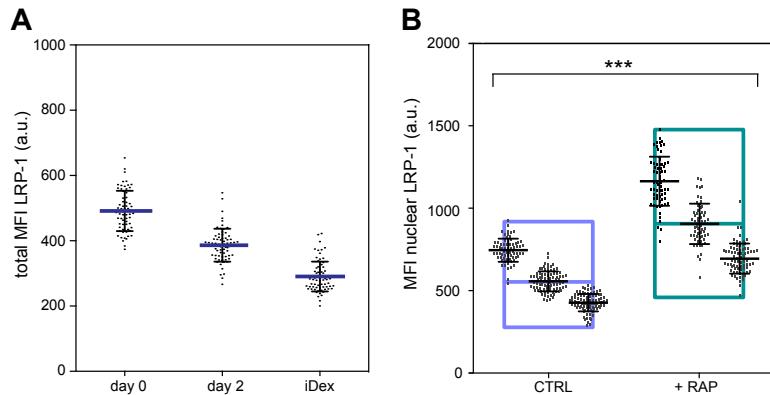
**(A)** Representative confocal image of permeabilized iDex DCs stained with a mouse monoclonal antibody against an extracellular epitope of MerTK. The dotted line represents the cell boundary, while the orange line represents the nuclear envelope. **(B)** Representative image of permeabilized iDex DCs stained with a mouse polyclonal antibody against the extracellular domain of MerTK. **(C)** Representative confocal image of a permeabilized tolerogenic DC directly isolated from the tumor environment of a cancer patient. The cell is stained for MerTK, and nuclear localization becomes apparent, while this is not the case for the isotype control. **(D)** Representative confocal image of a permeabilized THP-1 cell (monocytic cell line) stained for MerTK. HDAC-1 staining is used to identify the nuclear area, as it less evident from the transmission images in the case of rounded monocytes.



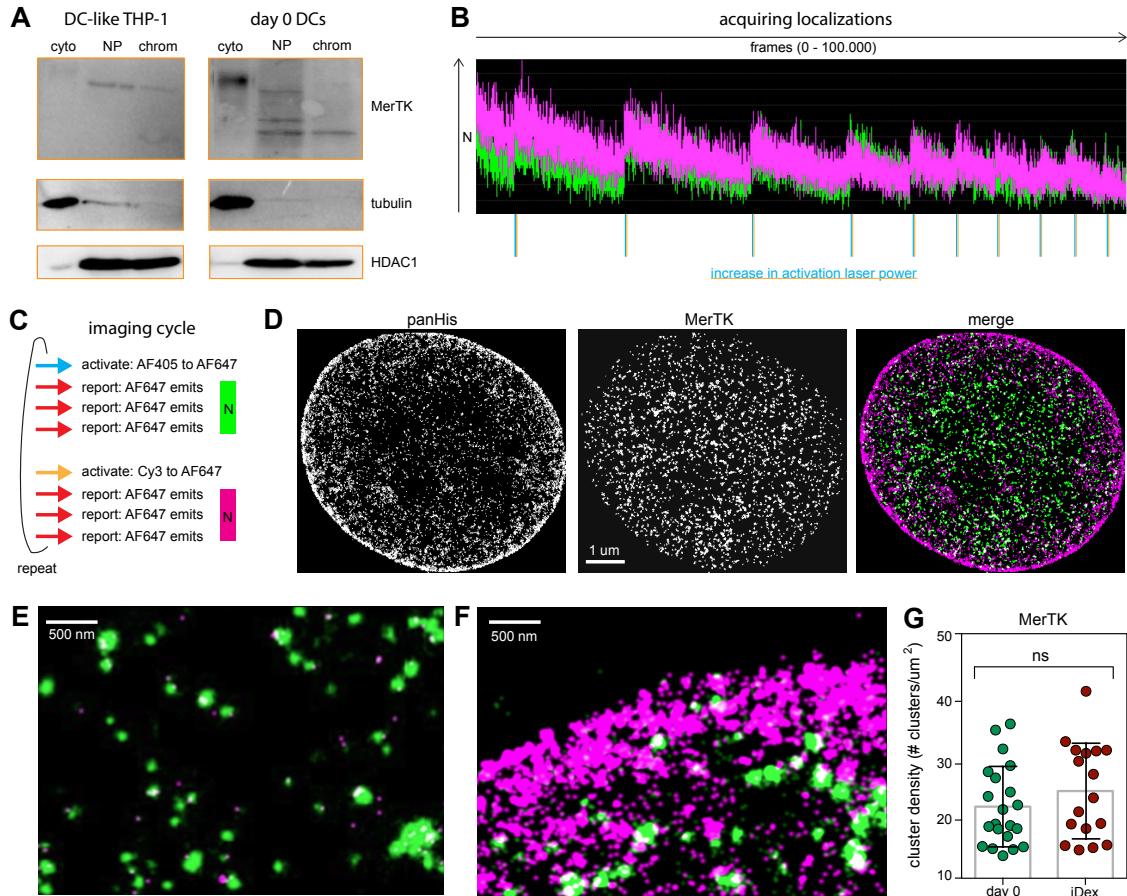
**Figure S3: Nuclear localization of MerTK in immune cell lines vs other tissue cell lines.**

**(A)** Representative confocal image of a permeabilized THP-1 cell (monocytic cell line) stained for MerTK. The nuclear area is identified using a HDAC-1 staining, since it is less clear from transmission images for the rounded monocytes. The dotted line represents the cell boundary; the orange line represents the nuclear envelope.

**(B)** Representative image of a permeabilized THP-1 cell that has been differentiated into a DC-like phenotype, stained for MerTK. The nuclear area is identified using HDAC-1 staining. **(C)** Representative image of a DC-like THP-1 stained for MerTK on the membrane. Nanoclustering of the receptor similar to that observed on the membrane of iDexs becomes apparent, as well as flattening and spreading of the cell. **(D)** Representative image of a MerTK transfected HeLa cell stained for MerTK on the membrane. Nanoclustering of the receptor on the membrane is observed, indicating successful transfection and correct incorporation of the transmembrane domain. **(E)** Representative image of a MerTK transfected HeLa cell, permeabilized and stained for MerTK intracellularly. No nuclear localization of MerTK is observed. **(F)** Representative image of permeabilized Hek293 cells stained for MerTK. Cells endogenously express MerTK, but it is not found in the nucleus. **(G)** Representative image of a Hek293 cell after transfection with MerTK to induce overexpression of the protein. Even though a clear increase in the intracellular MerTK level is obtained, nuclear localization is not observed.

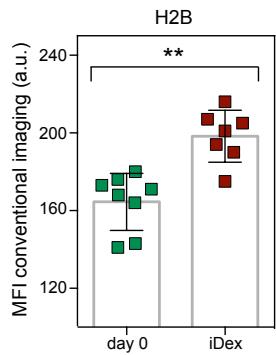


**Figure S4: Total LRP-1 expression levels** (A) MFI of LRP-1 in the entire cell (membrane - which did not exceed isotype control levels, cytosol and nucleus) over time in culture, at days 0, day 2 and iDexs (= day 4). Data from 3 donors. (B) MFI of nuclear LRP-1 with and without the addition of RAP, a ligand of LRP-1, to the culture. Monocytes were isolated normally, and RAP was added to one of the cultures after a few hours up to day 0, when the cells were harvested for imaging. Each spot represents a single nucleus; small plots in larger bars correspond to data from 3 different donors. Statistics was performed using the average value of each donor.



**Figure S5: Super resolution STORM imaging of MerTK and chromatin** (A) Representative Western blot showing the relative abundance of MerTK in different cellular fractions (cytoplasm, nucleoplasm, chromatin-bound) in both DC-like THP-1 and day 0 DCs. Tubulin and HDAC1 staining indicate correct fractionation of the cells. (B) Representative imaging trace during STORM acquisition. The time expressed in number of frames acquired is plotted against the number of localizations identified per frame (N). The vertical lines under the plot indicate when the activation laser power was increased, which is clearly seen in the peak increase in localizations registered. Over time the number of localizations is progressively reduced, indicating exhaustive imaging of all fluorophores. The traces also show that the number of localizations for both colours (MerTK in green and panHis in magenta) are comparable, validating the use of 2-color STORM imaging with minimal risk for bleedthrough. (C) Schematic representation of one imaging cycle: First, AF405 staining MerTK is activated by a pulse of the 405 nm laser line, and photons are transferred to the attached reporter dye AF647. Then, 3 pulses of the 647 nm laser line promote the emission of photons from AF647 and places the dye back into the dark state, until after the last pulse no localizations are recorded anymore. This makes the way free to start imaging the next colour without crosstalk. Cy3 staining panHis is activated by a pulse of the 561 nm laser line, and photons are transferred to attached reporter dye AF647. Then, 3 pulses of the 647 nm

laser line force the emission of photons from AF647, and places the dye back into the dark state, ready for the next imaging cycle. **(D)** Pixelated binary reconstructed STORM images of both panHis and MerTK, and the merge of both channels (green for MerTK and magenta for panHis). Pixel size corresponds to the position accuracy, namely 20 nm. These binary images are used to calculate the colocalization between both colours. Binary pixelated images are required for this correlation analysis since raw localizations with an exact  $x,y$  positions will never perfectly colocalize and are therefore not suitable for determination of the degree of colocalization between MerTK and panHis on a pixel to pixel basis. **(E)** Representative reconstructed STORM image of a cytosolic area in which no colocalization between MerTK and panHis is expected, as panHis labels the nucleus. MerTK vesicles show signal almost exclusively resulting from the AF405/AF647 dye pair (with only 2% of crosstalk with the panHis signal). **(F)** Representative reconstructed STORM zoom-in image on the nuclear periphery where the signal arises almost exclusively from we find the Cy3/AF647 dye pair labelling panHis. **(G)** Quantification of the number of MerTK nanoclusters per  $\mu\text{m}^2$  in the nucleus of both day 0 DCs and iDex DCs. No significant difference is observed. The data was obtained from the MerTK channel of the dual colour STORM images. Each dot corresponds to an individual nucleus. Data from 3 different donors. The average value per cell is depicted.



**Figure S6: Expression levels of H2B in the nucleus of day 0 vs iDex DCs. (A) MFI of nuclear H2B in day 0 vs iDex DCs.**  
Conventional images were taken before STORM acquisition of the same cells. This shows, together with Figure 5E,F, that the global increase in localizations per nanocluster and nanocluster density is indeed also reflected by a total increase of expression levels of H2B in the nucleus.

Sequence	Start	End	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	piskacek.org
ELVVVNYIAK	544	552	+	+	+	+	+	+	+	+	+	+	+	+	Perfect match
DELYEIMYS	827	835	+	+	+	+	+	+	+	+	+	+	+	+	Perfect match

**Figure S7: Prediction of 9aaTAD domains in MerTK sequence.** Prediction of putative 9aaTAD domains in the aminoacid sequence of MerTK. Prediction was performed using an algorithm developed by Piskacek et al (Piskacek et al., 2016, 2007). The algorithm was accessed through their website Piskacek.org and the settings recommended for mammalian cells were applied (moderately stringent pattern).