

## Mutational analysis of the DOK2 haploinsufficient tumor suppressor gene in chronic myelomonocytic leukemia (CMML).

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1 **Mutational analysis of the *DOK2* haploinsufficient tumor suppressor gene in**  
2 **chronic myelomonocytic leukemia (CMML)**

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4 Letter to the Editor  
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8 *Downstream of tyrosine kinases* (DOK) proteins are substrates of protein tyrosine  
9 kinases, acting as negative regulators of cell signaling pathways. <sup>1</sup> Loss of *DOK2*  
10 gene expression has been detected in human lung adenocarcinomas, and mice with  
11 *Dok2* haploinsufficiency develop lung cancers. <sup>2</sup> Mice lacking both *Dok1* and *Dok2*  
12 genes (the two first described *Dok* gene family members) present a  
13 myeloproliferative chronic myelogenous leukemia (CML)-like syndrome. <sup>3, 4</sup>  
14 Moreover, genetic ablation of *Dok* genes in a *BCR/ABL* transgenic background  
15 accelerates the apparition of the blastic crisis and leukemia induced by the BCR-ABL  
16 fusion oncoprotein, <sup>3, 4</sup> DOK1 and DOK2 adaptor proteins attenuate RAS/ERK and  
17 PI3K/AKT dependent-signaling pathways involved in myeloid cell proliferation. <sup>3-5</sup>  
18 Based on these studies, animal models <sup>2-4</sup> and data from solid tumors, <sup>2, 6</sup> *DOK*  
19 genes are now considered as tumor suppressors. However, the mutation status of  
20 *DOK1* and *DOK2* genes in patients with a chronic myeloproliferative neoplasm  
21 (MPN) remains to be defined.

22 Mutations in cell signaling genes have been reported in MPNs. <sup>7</sup> Chronic  
23 myelomonocytic leukemia (CMML) belongs to the MPN class. <sup>8</sup> Upon white blood  
24 cells (WBC) count CMML has been subdivided in myelodysplatic (MD-CMML) and

25 myeloproliferative (MP-CMML) subtypes. These two subtypes are associated with  
26 different gene expression profiles.<sup>9</sup>

27 Under informed consent, we analyzed gene mutations in bone marrow samples from  
28 30 MD-CMML and 36 MP-CMML patients (**Table S1**). Expectedly, mutations  
29 previously reported in CMML patients (such as *NRAS*, *CBL*, *PTPN11*, *FLT3*, *JAK2*,  
30 *NF1* genes)<sup>7</sup> were present in our MP-CMML cohort. We analyzed somatic mutations  
31 in *DOK1* and *DOK2* genes. Genomic DNA from bone marrow (BM) cells was  
32 amplified with 6 primer pairs covering the entire coding region (exons 1-5) of each  
33 *DOK* gene (**supplementary material**). We identified point mutations in the two *DOK*  
34 genes. For *DOK1*, two variants were found; L60Q in exon 1 coding for a functional  
35 protein-lipid interaction domain, a pleckstrin homology (PH) domain<sup>10</sup> and D263E in  
36 exon 5. For *DOK2*, four variants (2 x R201H, L238P, R215H) were found in 3/66  
37 CMML and 1/2 unclassified-myeloproliferative myelodysplastic neoplasm (U-  
38 MPN/MDS). These *DOK2* point mutations are located in exon 4 and the 5' end of  
39 exon 5, which code for the phosphotyrosine-binding (PTB) domain of the DOK  
40 protein.<sup>11</sup> Sorted CD3<sup>+</sup> lymphocytes of peripheral blood from *DOK* variant patients  
41 were only available for the MP-CMML patient with *DOK2* L238P mutation. The *DOK2*  
42 L238P mutation was present in myeloid cells but not in lymphoid cells (**Figure 1a**).

43 A three-dimensional (3D) structure model revealed that the L238P substitution would  
44 alter the structure of the DOK2 PTB domain, resulting in a loss of stable binding to  
45 phosphotyrosyl peptides (**Figure 1b**).

46 DOK2 binds via its PTB domain to tyrosine phosphorylated DOK1 protein.<sup>12</sup> Two  
47 arginine residues in positions 200 and 201 in the DOK2 PTB domain are critical for  
48 the direct interaction with the DOK1 molecule. The double DOK2 RR200-201AA  
49 mutant shows reduced PTB domain ligand binding.<sup>12</sup> We used this RR200-201AA

50 (RR) DOK2 mutant as a loss-of-function control. To test the ability of the L238P  
51 DOK2 mutant to bind to DOK1, KG-1 myeloid cells were transfected with different  
52 GFP-tagged DOK2 constructs. These cells were then treated with the potent tyrosine  
53 phosphatase inhibitor sodium pervanadate (pV), which causes tyrosine  
54 phosphorylation of the major cellular proteins (**Figure 1c**). Endogenous DOK1 was  
55 immunoprecipitated from cell lysates and the GFP-DOK2 fusion proteins were  
56 revealed by GFP Western-blot. A GFP signal in DOK1 immunoprecipitates was  
57 detected only when a wild-type (WT) version of DOK2 but not when DOK2 L238P  
58 mutant protein was transfected (**Figure 1c**).

59 The DOK2 PTB domain plays a role in the negative regulation of signaling events in  
60 myeloid cells.<sup>13</sup> PTB-deficient DOK2 molecules lose their inhibitory effects on  
61 functional cell events in lymphoid cells.<sup>12</sup> Via its interaction with RAS GTPase  
62 activating protein (RasGAP), a potent inhibitor of RAS, DOK2 acts as a negative  
63 regulator of the RAS/ERK signaling pathway in myeloid cells.<sup>1, 5, 13</sup> To test the  
64 capacity of the L238P DOK2 mutant to attenuate extracellular signal-regulated  
65 kinases-1/2 (ERK-1/2) activation, KG-1 cells were transfected with different GFP-  
66 tagged DOK2 constructs. These cells were then treated or not with pV and ERK-1/2  
67 activation was detected by phospho-ERK Western-blot (**Figure 1d**). DOK2 WT  
68 overexpression reduced pV-induced ERK phosphorylation. In contrast, the DOK2  
69 L238P mutant was unable to inhibit ERK activation, suggesting that it could be  
70 considered as a loss-of-function mutant.

71 The major characteristic of MP-CMML is an abnormally high rate of cell proliferation.  
72 <sup>14</sup> We designed a cell proliferation assay using WT and DOK1/DOK2-deficient mouse  
73 embryonic fibroblasts (MEF) (**Figure 1e**). In DOK-deficient cells (**Figure 1e**, right  
74 panel), DOK2 WT but not its PTB-deficient mutants reduced autonomous cell

75 proliferation. Moreover, expression of DOK2 L238P mutant in WT MEF induced a  
76 hyperproliferation (**Figure 1e**, left panel). These results are in agreement with the MP  
77 phenotype of the CMML patient harboring a *DOK2* L238P mutation.

78 The possibility that DOK2 gene expression is downregulated or mutated in MPN has  
79 been previously suggested.<sup>15</sup> Here, we have shown that point mutations in *DOK1*  
80 and *DOK2* genes are detected with low frequency in CMML, but that they may have  
81 consequences for the function of the DOK2 PTB domain.

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85 **Conflict of interest**

86 The authors declare no conflict of interest

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102 **Author contributions**

103 EC, VG-B, DB and JAN designed the experiments and wrote the manuscript. EC, XM  
104 NC and AM performed and analyzed the data. P-PP provided important materials.

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124 Supplementary information is available at Leukemia's website

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196 **Figure legend**

197 **(a) Identification of an acquired and heterozygous *DOK2* L238P point mutation**  
198 **in an MP-CMML patient.** Sequencing profile of *DOK2* MP-CMML patient (HD-1236).  
199 DNA samples were extracted from whole bone marrow or sorted CD3<sup>+</sup> T cells of  
200 peripheral blood. The HGVS nomenclature of the identified *DOK2* gene variation is  
201 c.713T>C (p.Leu238Pro) (Table S2).

202 **(b) *DOK2* L238P mutation alters the 3D structure of the *DOK2* PTB domain.** *Top:*  
203 Linear structure of the *DOK2* molecule, containing an N-terminal PH domain, a  
204 central PTB domain and several phosphorylable tyrosine (Y) residues in the C-  
205 terminal part. The L238P point mutation (red asterisk) is located in the PTB functional  
206 domain. *Bottom:* 3D structural model of the *DOK2* PTB domain (green ribbon)  
207 interacting with *DOK1* EMLENLpY phospho-peptide (red) complex. The L238P single  
208 mutation (grey) would affect the 3D conformation of the protein, bending the helix  $\alpha 2$   
209 (represented by light green arrows that illustrate the consequences of such mutation)  
210 thus preventing the clamp between the C-terminal part of the protein and the  
211 phospho-peptide.

212 **(c) *DOK2* L238P mutation induces a loss of *DOK2* heterodimerization with the**  
213 ***DOK1* protein.** KG-1 myeloid cells were transfected by nucleofection with plasmids  
214 coding for GFP-tagged wild-type (wt) *DOK2*, a control *DOK2* mutant with a loss-of-  
215 function in the PTB domain (RR200-201AA, RR), the L238P point mutant (L238P) or  
216 GFP alone (mock). KG-1 cells were treated with sodium pervanadate (pV) at 50  $\mu$ M  
217 for 5 min at 37°C. Cells were lysed and lysates were immunoprecipitated using an  
218 anti-Dok1 antibody followed by SDS-PAGE and anti-GFP western-blot (WB) allowing  
219 the identification of *DOK2* GFP-fusion proteins with the endogenous *DOK1* molecule.  
220 *DOK1* immunoprecipitates were controlled by *DOK1* WB. In parallel, whole cell

221 lysates (WCL) were separated by SDS-PAGE and blotted for GFP expression.  
222 Molecular weight markers were reported in the left size of the blots. This panel shows  
223 representative blots of 2 independent experiments.

224 **(d) The L238P mutant DOK2 molecule is unable to reduce pervanadate-induced**  
225 **ERK-1/2 phosphorylation in KG-1 myeloid cells.** KG-1 myeloid cells were  
226 transfected by nucleofection with plasmids encoding for GFP-tagged wild-type (WT)  
227 DOK2, a control DOK2 mutant with a loss-of-function in the PTB domain (RR200-  
228 201AA, RR), the L238P point mutant (L238P) or GFP alone (mock). KG-1 cells were  
229 treated or not with sodium pervanadate (pV) at 1  $\mu$ M for 5 min at 37°C. Whole cell  
230 lysates (WCL) were separated by SDS-PAGE and subsequently immunoblotted for  
231 phospho-ERK-1/2 (p-ERK1/2 WB). The blots were reprobed for  $\beta$ -tubulin expression  
232 as a loading control and GFP expression to detect the presence of GFP-DOK2 fusion  
233 proteins. Molecular weight markers were reported in the left size of the blots. This  
234 panel shows representative blots of 2 independent experiments.

235 **(e) Expression of the L238P mutant DOK2 molecule increases cell proliferation.**  
236 Mouse embryonic fibroblasts (MEFs) from wild-type (left: MEFs *wt*) or *Dok1-Dok2*  
237 double KO (right: MEFs *Dok DKO*) mice were infected by retroviruses expressing  
238 DOK2 expression constructs (pMIG alone, pMIG *Dok2 wt*; pMIG *Dok2 RR* for  
239 RR200-201AA and pMIG *Dok2 L238P*) and sorted for positive GFP expression.  
240 Absolute cell counts were performed by flow cytometry. Only DOK2 PTB loss-of-  
241 function mutants (L238P and RR) induce an increase of cell proliferation in MEFs *wt*  
242 compared to mock-infected cells. DOK2 *wt* is acting as a cell proliferation attenuator  
243 in the absence of endogenous DOK proteins (MEFs *Dok DKO*). \*  $P < 0.05$ ; \*\*\*  
244  $P < 0.005$  (two-way ANOVA test). The data are representative of 3 independent  
245 experiments.

