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

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Letter to the Editor

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

Mutations in cell signaling genes have been reported in MPNs. [7] Chronic myelomonocytic leukemia (CMML) belongs to the MPN class. [8] Upon white blood cells (WBC) count CMML has been subdivided in myelodysplatic (MD-CMML) and

myeloproliferative (MP-CMML) subtypes. These two subtypes are associated with different gene expression profiles. [9]

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

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

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

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

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

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

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

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

Conflict of interest

The authors declare no conflict of interest

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Author contributions

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

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Supplementary information is available at Leukemia's website

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Figure legend

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

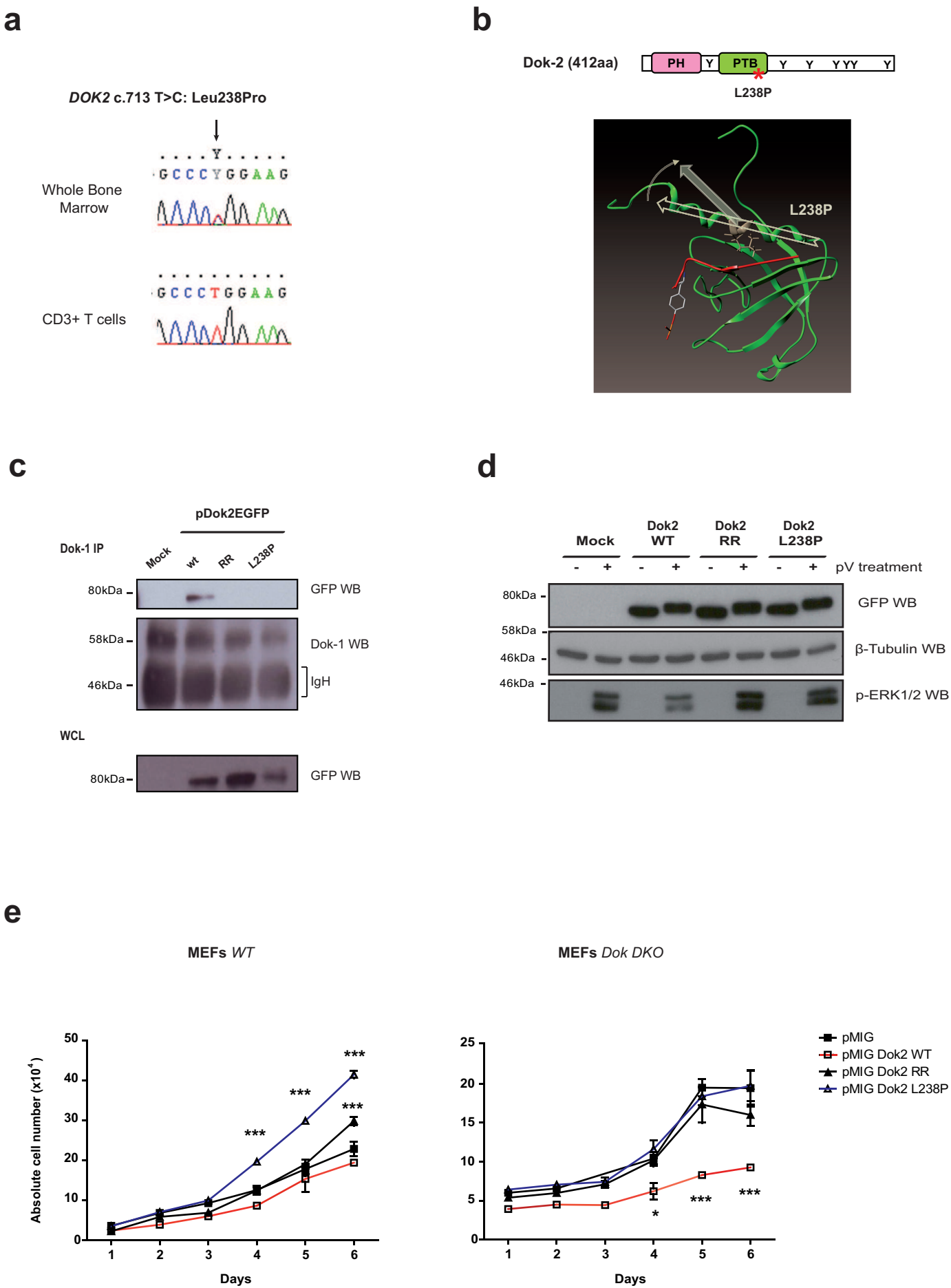
(b) *DOK2* L238P mutation alters the 3D structure of the DOK2 PTB domain. *Top*: Linear structure of the DOK2 molecule, containing an N-terminal PH domain, a central PTB domain and several phosphorylable tyrosine (Y) residues in the C-terminal part. The L238P point mutation (red asterisk) is located in the PTB functional domain. *Bottom*: 3D structural model of the DOK2 PTB domain (green ribbon) interacting with DOK1 EMLENLpY phospho-peptide (red) complex. The L238P single mutation (grey) would affect the 3D conformation of the protein, bending the helix α2 (represented by light green arrows that illustrate the consequences of such mutation) thus preventing the clamp between the C-terminal part of the protein and the phospho-peptide.

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

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

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

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


a
DOK2 c.713 T>C: Leu238Pro
Whole Bone Marrow
CD3+ T cells
b
Dok-2 (412aa)
PH
PTB
L238P
L238P
c
pDok2EGFP
Dok-1 IP
Mock
wt
RR
L238P
80kDa
GFP WB
58kDa
Dok-1 WB
46kDa
IgH
WCL
80kDa
GFP WB
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Mock
Dok2 WT
Dok2 RR
Dok2 L238P
pV treatment
80kDa
GFP WB
58kDa
β-Tubulin WB
46kDa
46kDa
p-ERK1/2 WB
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Absolute cell number (x10⁴)
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pMIG
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