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DOCK9 induces membrane ruffles and Rac1 activity in cancer HeLa epithelial cells

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ABSTRACT

Dedicator-of-cytokinesis (DOCK) proteins are a family of guanine-nucleotide exchange factors (GEF) for Rho GTPases. The DOCK-D homology subfamily comprises DOCK9, DOCK10, and DOCK11. DOCK9 and DOCK11 are GEFs for Cdc42 and induce filopodia, while DOCK10 is a dual GEF for Cdc42 and Rac1 and induces filopodia and ruffles. We provide data showing that DOCK9, the only one of the DOCK-D members that is not considered hematopoietic, is nevertheless expressed at high levels in T lymphocytes, as do DOCK10 and DOCK11, although unlike these, it is not expressed in B lymphocytes. To investigate DOCK9 function, we have created a stable HeLa clone with inducible expression of HA-DOCK9. Induction of expression of HA-DOCK9 produced loss of elongation and polygonal shape of HeLa cells. Regarding membrane protrusions, HA-DOCK9 prominently induced filopodia, but also an increase of membrane ruffles. The latter was consistent with an increase in the levels of activation of Rac1, suggesting that DOCK9 carries a secondary ability to induce ruffles through activation of Rac1.

1. Introduction

Dedicator-of-cytokinesis (DOCK) designates a group of 11 homologous proteins that act as guanosine nucleotide exchange factors (GEFs) for Rho GTPases [1,2]. Through this activity, DOCK proteins play roles in actin cytoskeleton dynamics, cell adhesion and movement. The DOCK-D or Zizimin subfamily is composed of 3 members, DOCK9, DOCK10, and DOCK11. DOCK9 and DOCK11 interact *in vitro* mainly with Cdc42 [3–6], act as GEFs for Cdc42 [3–5], and induce filopodia when transiently expressed in adherent cells [3–7]. We have previously generated a stable HeLa clone with inducible expression of DOCK10 [6], which was instrumental to demonstrate a role for DOCK10 as a dual GEF for Rac1 and Cdc42, also demonstrated by Jaudon et al. [8], and in induction of ruffles and filopodia. Consistent with their cell remodelling function, DOCK9 and DOCK10 have been related to glioblastoma and breast cancer invasion, respectively [9,10].

DOCK10 and DOCK11 are expressed at its highest levels in circulating leukocytes, whereas DOCK9 is widely distributed in organs, but not prominently in hematopoietic tissue [11,7]. In steady-state conditions, DOCK10 is highly expressed in circulating T and B cells [11,12], and DOCK11 is expressed at similar levels in murine T and B splenocytes [4].

In this paper, we have investigated expression of DOCK9 and DOCK11 in human hematopoietic cell subsets. In addition, we have generated a stable HeLa clone with inducible expression of DOCK9 in order to gain further insights into the role of DOCK9 in cell morphology and GEF function.

2. Materials and methods

2.1. Human samples

Whole blood samples from 5 volunteer donors and 12 chronic lymphocytic leukemia (CLL) patients, and spleen samples from 5 cadaveric transplant donors, were previously used [12]. The study was approved by the Institutional Review Board of the Hospital Virgen de la Arrixaca and written informed consent was obtained according to the Helsinki Declaration guidelines. CLL B cells were cultured in RPMI-1640 medium with 10% fetal calf serum (Biowhittaker, Cambrex, East Rutherford, NJ), 50 U/mL penicillin, 50 U/mL streptomycin, 2,5 μg/mL amphotericin B, and 2 mM L-glutamine ('RPMI-C', where 'C' means 'complete') and 10 ng/mL human recombinant IL-4 (BD Pharmingen, BD Biosciences, San Diego, CA).

Abbreviations: DOCK, dedicator-of-cytokinesis; dox, doxycycline; GEF, guanine-nucleotide exchange factor; GST, glutathione S-transferase; HA, hemagglutinin; PAK1, p21-activated kinase 1; PBD, p21-binding domain; tTA, tetracycline transactivator; wt, wild type

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2.2. QRTPCR

QRTPCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). For primary samples, the following the following Applied Biosystems Taqman assays from were used, as previously described [12]: a predesigned assay directed to exon boundary 27–28 of DOCK9 (Hs_00324508_m1); a custom assay directed to exon boundary 1–2 of DOCK11 forward 5′- CCGGCAGAGCGTGTCT -3′, reverse 5′- TGGGCAATAACATTCTCATAGTCCA -3′, probe (FAM) 5′- TTGGCCTTTTCCAGCACCAC -3′; and a predesigned GAPDH assay (Hs_99999905_m1) for normalization parameter for relative quantitation.

2.3. Northern blot analysis

Northern blot analysis was performed as previously described [11]. Briefly, blots were hybridized using [α-^{32}P]dCTP labelled probes for DOCK9, DOCK10, DOCK11 and GAPDH, then washed and exposed to storage phosphor screens, which were scanned in a Typhoon 9210 Variable Mode Imager (GE Healthcare).

2.4. Generation of stable cell clones with regulatable expression

Stable clones with regulatable HA-DOCK9 expression of HeLa cells were generated using the tet-off system following a procedure previously described [6]. Briefly, the doxycycline (dox)-repressible HeLa-tetracycline-transactivator (tTA) cell clone, grown in Dulbecco's minimum essential medium (DMEM) with the same supplements (DMEM-C), was transfected using lipofectamine reagent (Invitrogen, Carlsbad, CA) with pJEF4-HA-DOCK9 plasmid, which drives expression of human influenza hemagglutinin (HA)-tagged DOCK9 under the tTA-inducible promoter (generated by PCR cloning of DOCK9 into pJEF4) (Supporting information Table S1). Selection was performed with puromycin, G418, and dox. Colonies were checked for HA-DOCK9 expression in the absence of dox by western blot analysis. The HeLa-tTA cell clone was also transfected with pJAG2-EGFP-Rac1N17 or with pJAG2-EGFP-Rac1wt (generated by PCR cloning from plasmids published in Subauste et al. [13], into pJAG2), which drive expression of the EGFP-tagged Rac1, dominant negative or wild-type (wt), under the tTA inducible promoter, followed by selection with puromycin, zeocin and dox. Positive colonies were detected by green fluorescence and checked for expression of EGFP and Rac1 in the absence of dox by western blot analysis.

2.5. Western blot analysis

Immunoreactive proteins were detected as previously described [6,12]. Briefly, SDS-PAGE-fractionated proteins, electroblotted onto nitrocellulose membranes, were incubated with the indicated Abs (Supporting information Table S2), detected by chemiluminescence, and analyzed in a Molecular Imager ChemiDoc™ XRS+ (Bio-Rad Laboratories, Hercules, CA).

2.6. Microscopy

HeLa cells were grown on to 12 mm BioCoat Poly-L-Lysine-coated coverslips (Corning Inc., Corning, NY). Preparations were labelled using the F-actin visualization biochem kit (Cytoskeleton Inc., Denver, CO), as previously described [6]. Cells were examined and analyzed in an Eclipse T*i* inverted microscope (Nikon Instruments Inc., Melville, NY). The rate of cell elongation was measured as the ratio between maximum and minimum Feret diameters. Values taken by this ratio are ≥ 1, and increase as the cells are more elongated. Phase contrast time lapse images of cells grown on to poly-L-lysine-coated coverslips were acquired as previously described [6].

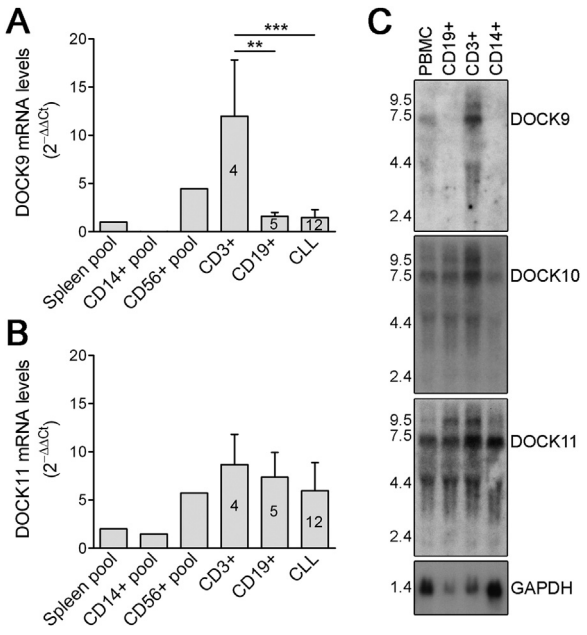

Fig. 1. Expression levels of DOCK-D mRNAs in hematopoietic cell subsets. (A-B) Relative quantitation by QRTPCR of the mRNA levels of DOCK9 (A) and DOCK11 (B) in hematopoietic cell subsets. Mean ± S.D. of the indicated number of samples; **, $p < 0.01$, Student's *t*-test. (C) Northern blot analysis in a representative individual. Blots were consecutively hybridized with DOCK10, DOCK9, DOCK11, and GAPDH ^{32}[P]-labelled probes, washing the blots between hybridisations. The positions of the size markers are indicated in kb to the left.

2.7. Rac1 activation assays

Replicate aliquots of the HeLa clones with regulatable expression of HA-DOCK9 cultured for 24 h in the presence and absence of dox were assayed by pulldown using glutathione S-transferase (GST)-p21-activated kinase 1 (PAK1)-p21-binding domain (PBD) (GST-PAK1-PBD) bound beads, as previously described [6]. HeLa clones expressing EGFP-tagged Rac1wt, Rac1N17 and Rac1L61 cultured for 24 h in the absence of dox were also assayed as control experiments.

3. Results and discussion

3.1. DOCK9 is expressed in steady-state circulating human T cells

The levels of expression of DOCK9 and DOCK11 in human hematopoietic cell subsets were measured by QRTPCR. Unexpectedly, DOCK9 was found to be highly expressed in circulating CD3+ T cells (Fig. 1A). In contrast, as anticipated, DOCK11 mRNA levels were found similarly high in circulating T and B cells (Fig. 1B). Therefore, the DOCK10 and DOCK11 profiles resemble. Northern blot analysis from a representative individual reproduced these observations (Fig. 1C). These results indicate that DOCK9 must play a role in human T lymphocytes.

3.2. HA-DOCK9 induces filopodia, but also membrane ruffles, in HeLa cells

To define the role of DOCK9 in cell morphology, we have generated stable HeLa clones with inducible expression of HA-DOCK9. The best expressor clone in the absence of doxycycline (dox) was HA-DOCK9 C6, which was also tightly repressed in the presence of dox, as assessed by Western blot analysis (Fig. 2A). Immunofluorescence analysis using this clone showed that under repression by dox, cells retained the characteristic elongated polygonal morphology of HeLa cells, with presence of edge stress fibers detected by phalloidin binding to F-actin (Fig. 2B, showing 3 representative cells). HA-DOCK9 induced loss of elongation

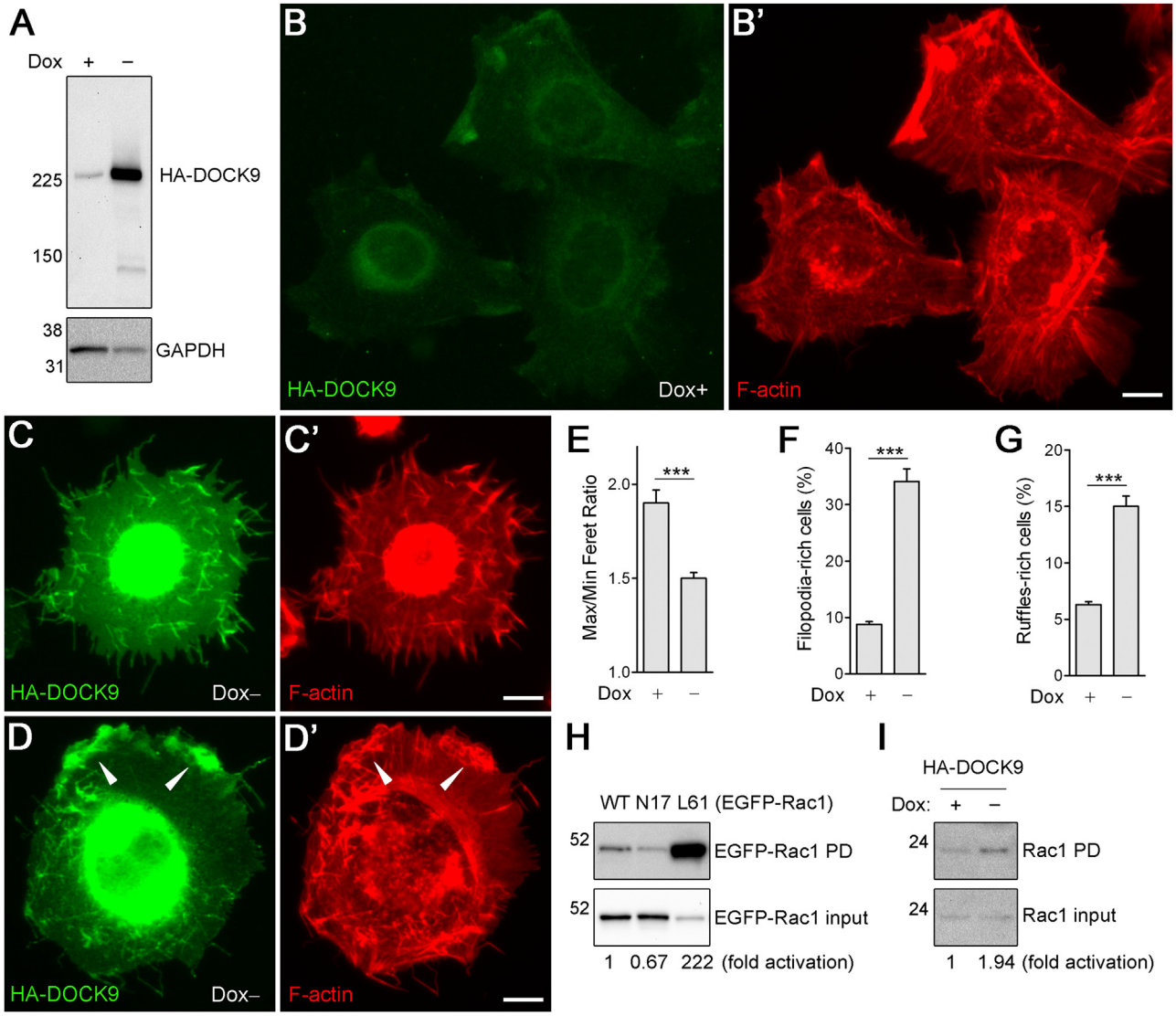

Fig. 2. Changes in morphology, membrane protrusions and activation of Rac1 induced by expression of HA-DOCK9 in HeLa cells. (A) Western blot analysis for inducible expression of HA-DOCK9 in HeLa cell clone C6 using anti-HA Ab, and anti-GAPDH Ab as a loading control. Total protein extracts were performed 24 h after washing the cells free of dox and reseeding with (+) or without (−) 2 ng/mL dox. (B-D, B′-D′) Immunofluorescence microscopy representative examples of HeLa HA-DOCK9 clone cultured as detailed in A onto poly-lysine-coated coverslips and labelled with HA-FITC (B-D), and/or phalloidin-TRITC (B′-D′), in the presence (B, B′) or in the absence of dox (C, C′, D, D′). Arrowheads in D, D′ depict membrane ruffles. Scale bars, 10 μm. (E) Max/min Feret ratio of HeLa HA-DOCK9 clone cultured as detailed in A, indicating a significant loss of cell elongation induced by expression of HA-DOCK9 (G-H) Proportion of filopodia-rich cells (F) and ruffles-rich cells (G) in HeLa HA-DOCK9 clone cultured as detailed in A, indicating a significant increase in filopodia and ruffles induced by expression of HA-DOCK9. E to G charts depict mean ± S.E.M; ***, $p < 0.001$, Student's *t*-test. (H) GST-PAK1-PBD pulldown assay for activation of Rac1 in EGFP-Rac1wt, dominant negative EGFP-Rac1N17 (negative control), and constitutively active EGFP-Rac1L61 (Ruiz-Lafuente, 2015; positive control) HeLa clones following 24 h in the absence of dox. Anti-EGFP was used as primary Ab. A representative experiment, out of 4 performed, is shown. PD, pulldown. (I) GST-PAK1-PBD pulldown assay for activation of endogenous Rac1 in HeLa HA-DOCK9 following 24 h in the presence and absence of dox. Anti-Rac1 was used as primary Ab. A representative experiment, out of 4 performed, is shown. The positions of the size markers are indicated in kDa to the left in A, H, and I.

and of stress fibers (Figs. 2C and D, showing 2 representative cells; and Fig. 2E, showing Max/Min Feret Ratio as an elongation index). Concomitantly, HA-DOCK9 induced a strong increase in the number of cells rich in filopodia (Fig. 2C, and Fig. 2F), and a milder, but significant increase in the number of cells rich in membrane ruffles (Fig. 2D, showing a cell that develops filopodia and ruffles concurrently, Supporting Information Movie 1, and Fig. 2G).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.bbrep.2018.05.004.

Induction of filopodia by DOCK9 was first demonstrated using microinjection experiments in NIH-3T3 cells [3]. Involvement of activation of Cdc42 and Rac1 in filopodia and ruffles formation, respectively, are firmly established concepts in cell biology [14–17], and they have also been proven in HeLa cells with inducible expression of constitutively active EGFP-tagged Cdc42L61 and Rac1L61, respectively [6]. However, a role for DOCK9 in ruffle formation had not been previously reported.

3.3. HA-DOCK9 induces activation of Rac1

To test the potential involvement of Rac1 in induction of ruffles by HA-DOCK9, we performed PAK1-PBD activation assays. HeLa cells provide a cellular context of low baseline activation of Rac1, as indicated by the little difference observed in the levels of Rac1 activation between EGFP-Rac1wt and EGFP-Rac1N17 clones (Fig. 2H). Analysis of the inducible HA-DOCK9 clone showed activation of endogenous Rac1

(Fig. 2I).

Our previously published data showed that DOCK9 interacts *in vitro* with nucleotide-free Rac1, suggesting a potential role of DOCK9 as a Rac1 GEF, though these results were not highlighted because interaction was weak compared to that between DOCK9 and Cdc42 [6]. The new data presented here suggest that DOCK9, beyond its ability to induce filopodia through activation of Cdc42, also carries a secondary ability to induce ruffles through activation of Rac1. The stable DOCK9 inducible transfectant cell line, together with our previously published DOCK10.1 cell line, will be valuable for investigating the functions of these regulators of small GTPases in different environments. Comparison of both models suggests that DOCK9 has a more prominent role in the formation of filopodia and DOCK10 in the formation of ruffles.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.05.004.

Appendix B. Supporting information

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