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BRIEF REPORT

Mechanism of cell-intrinsic adaptation to Adams-Oliver Syndrome gene *DOCK6* disruption highlights ubiquitin-like modifier ISG15 as a regulator of RHO GTPases

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ABSTRACT

DOCK6 is a RAC1/CDC42 guanine nucleotide exchange factor, however, little is known about its function and sub-cellular localization. DOCK6 regulates the balance between RAC1 and RHOA activity during cell adhesion and is important for CDC42-dependent mitotic chromosome alignment. Surprisingly, a cell intrinsic adaptation mechanism compensates for errors in these DOCK6 functions that arise as a consequence of prolonged DOCK6 depletion or complete removal in *DOCK6* knockout cells. Down-regulation of the ubiquitin-like modifier ISG15 accounts for this adaptation. Strikingly, although most other DOCK family proteins are deployed on the plasma membrane, here we show that DOCK6 localizes to the endoplasmic reticulum (ER) in dependence of its DHR-1 domain. ER localization of DOCK6 opens up new insights into its functions.

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The Rho Family GTPases RAC1, CDC42 and RHOA are involved in many critical signaling pathways. They regulate actin polymerization, actin filament bundling, cell polarity, motility, differentiation, cell cycle phases, gene transcription and cellular transformation.[1-3] RHOA is required for actomyosin contractility to promote cell rounding, while RAC1 induces actin polymerization to form elongated cellular protrusions in interphase cells.[4] These impacts make the fine balance and precise spatio-temporal regulation of these Rho GTPases an essential aspect of cellular life.[5,6]

Guanine nucleotide exchange factors (GEFs) promote and regulate RHO GTPase activity by promoting the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP).[1] DOCK6 is a member of an atypical family of GEFs called Dedicator of Cytokinesis.[7,8] As for other DOCK GEFs, DOCK6 shares 2 conserved domains, the DHR-1 and DHR-2 motifs (Fig. 1A). The DHR-2 domain has been shown to harbor the GEF activity, while DHR-1 exhibits weak homology to the C2 domain lipid-binding module.[7] Recessive mutations that lead to inactivation of the *DOCK6* gene are one cause of Adams-Oliver Syndrome (AOS). AOS is usually characterized by an abnormality in scalp skin development and terminal transverse limb defects.[9-11] However, many additional organs are affected in AOS patients. Earlier studies indicated that, *in vitro*, DOCK6 can act as a dual specific GEF for RAC1 and CDC42.[8] In mice DOCK6 is highly expressed in dorsal root ganglion neurons where it mainly acts as a RAC1 GEF. DOCK6 depletion in mice impairs axon growth and branching as AKT1 kinase and PP2A phosphatase reciprocally regulate DOCK6 activity.[12]

Many DOCK-family members are associated with the plasma membrane and cytosol.[7,13,14] Interestingly, stable expression of the LAP (Localization Affinity Purification) tagged full length (FL) DOCK6 showed that DOCK6 co-localized with a marker for the endoplasmic reticulum (ER); RFP-KDEL[15] (Fig. 1A and B). The localization of DOCK6 truncation constructs indicated that the DHR-2 domain was dispensable for this ER association yet an intact DHR-1 domain of DOCK6 was essential (Fig. 1A and B). Nevertheless, the DOCK6 DHR-1 domain alone did not direct localization to ER to indicate that although the DHR-1 domain is required for ER localization it is probably not sufficient. However, we cannot rule out the possibility of the truncation inducing a protein-folding defect that leads to mis-localization (not on ER) of this DOCK6 DHR-1 domain. DHR-1 domains of some DOCK-family proteins interact with phosphatidylinositol (PtdIns) (3,5)-bisphosphate and PtdIns(3,4,5)P_3 signaling lipids.[7,13,14] Presence of PtdIns (3,4,5)P_3 signaling lipids not only on plasma membrane, but also on the endomembrane system, may assist recruitment of DOCK6 to the ER.[16] Moreover, SPIKE1, the only DOCK-family member in plants, is associated with ER exit sites in *Arabidopsis*.[17]

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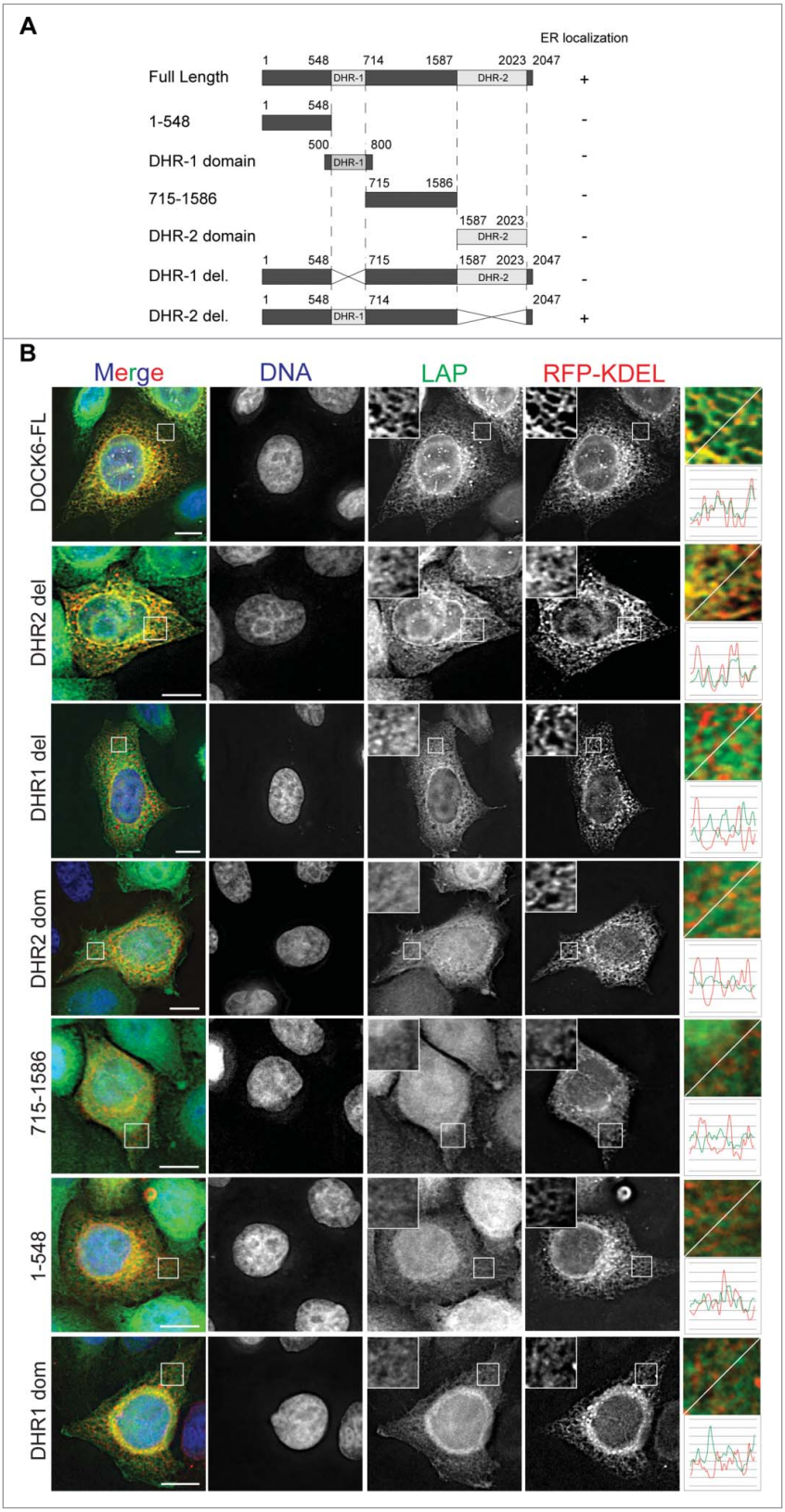

Figure 1. DOCK6 localizes to ER and DHR-1 domain is essential for this localization. (A) Summary of the ER localization of the constructs used in (B). (B) ER localization of DOCK6. Clonal HeLa cell lines stably expressing the indicated proteins in a doxycycline inducible manner were transfected with the *RFP-KDEL* construct for ER labeling. Co-localization of the indicated LAP tagged DOCK6 constructs (green) and the RFP-KDEL ER marker (red) was analyzed. White insets in the LAP and RFP-KDEL panels are shown as overlaps on the right hand side of the figure. Line scans along the white line in the inset testing co-localization of the LAP-tagged protein and RFP-KDEL are shown below. Scale bars, 10 μm.

The interphase polygonal ER network in the peripheral cell region disappears as soon as cells enter mitosis.[18] The mitotic ER is entirely excluded from the spindle region, with the exception of small fraction of cisternal ER that associates with spindle poles.[18] Throughout prophase and metaphase DOCK6 maintained its co-localization with RFP-KDEL marked ER including the population at the spindle poles (Fig. 2). Localization at peripheral regions of the cell persisted throughout anaphase until DOCK6 localization returned to the reforming nuclear envelope in telophase[18] (Fig. 2). DOCK6 did not associate with the midbody or the cytokinesis site.

Since little was known about the function of this GEF in non-neuronal cells, we conducted siRNA depletion experiments with HeLa cells. These acute knockdown (siRNA) experiments indicated that DOCK6 depletion induced the majority of the cells to round up and to cover a diminished area, extensive plasma membrane blebbing and collapse of the actin, microtubule and vimentin networks.[19] We found DOCK6 to be an important factor in mediating the balance between RAC1 and RHOA activities that promote cell spreading during interphase.[19] RAC1 and RHOA GTPases can affect each other's activities. This reciprocal inhibitory crosstalk between them regulates cell morphology.[5,20,21]

Rho GTPases are also involved in mitotic progression; increasing RHOA activity is required for the formation of a contractile ring during cytokinesis. Local activation of RAC1 at centrosomes by the GEF Tiam-1 is important for centrosome separation and bipolar spindle formation.[22-24] CDC42 has also been reported to have vital functions in mitosis. A peak in CDC42 activity in metaphase is essential for stable, bi-oriented attachment of spindle microtubules to the kinetochores leading to the proposal that the formin mDia3 interacts with GTP bound form of CDC42 to form a protein complex with the histone H3-like centromeric protein A (CENP-A) that localizes in close proximity of $p150^{Glued}$ (a dynein-dynactin complex component) at the kinetochores of the chromosomes, thereby stabilizing bi-oriented microtubule attachments.[22,25-27] Additionally, GEFs and GAPs such as DLC2, Ect2 and MgcRacGAP have been reported to regulate metaphase activity of CDC42.[22,27-29] Given this striking impact of CDC42 upon chromosome alignment and the fact that DOCK6 is a GEF for CDC42, we conducted live cell imaging experiments with synchronized HeLa cells to reveal that the reduction in CDC42 activity when DOCK6 is depleted led to a failure of alignment on the metaphase plate. This caused cell cycle arrest in pro-metaphase or metaphase with misaligned chromosomes.[19]

Although a sub-fraction of AOS patient fibroblasts resemble the interphase phenotypes arising from DOCK6 siRNA depletion (spreading defect and

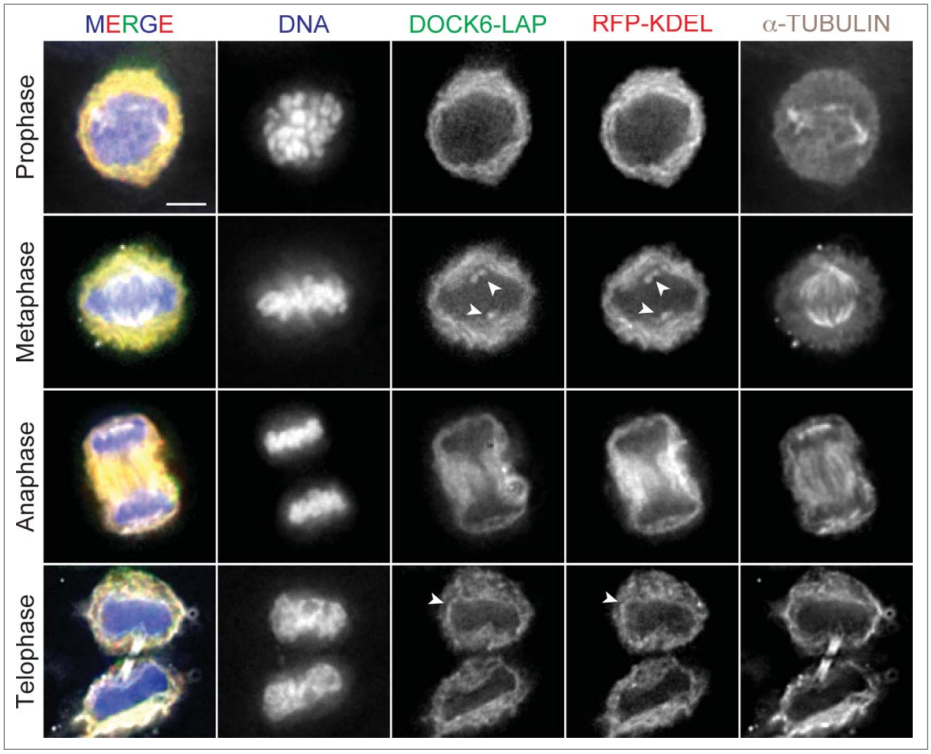

Figure 2. Mitotic co-localization of ER marker RFP-KDEL and LAP DOCK6. White arrowheads on metaphase and telophase panels indicate co-localization of RFP-KDEL and DOCK6-LAP on spindle poles and reforming nuclear envelope, respectively. Scale bar, 10 μm.

membrane blebbing), their phenotypes were not as severe as those induced by the acute depletion of DOCK6.[9,19] We confirmed the specificity of the DOCK6 depletion regime and the requirement for the DHR-2 GEF domain within DOCK6 for the rescue. We found that prolonged siRNA depletion of DOCK6 (120 h) led to a milder cytoskeletal phenotype than we observed upon a standard 48 h regimen of acute depletion.[9,19] In addition, *DOCK6* KO HeLa cells created with either Zinc Finger Nuclease (ZFN), or Clustered Regularly Interspaced Short Palindromic repeats/CRISPR Associated System-protein 9 (CRISPR/Cas9) systems, also showed milder spreading and actin cytoskeleton phenotypes, reminiscent of those seen in AOS patient fibroblasts.[9,19] Moreover, no mitotic progression defects were observed in either *DOCK6* KO cells or in AOS patient fibroblasts.[9,19] Taken together, these data suggested an adaptation mechanism that compensates for loss of DOCK6 function.

To solve the basis for this enigma, and to identify the factors that compensate for the disruption of DOCK6 activity, we compared the expression profiles of 2 independent *DOCK6* KO HeLa clonal cell line models with wild type control cells. mRNA profiles of an AOS patient fibroblasts which have a homozygous 4 base pair deletion in the *DOCK6* gene (c.1362_1365delAACT, p.Thr455Serfs*24; RefSeq accession number: NM_020812.2) with 2 healthy age-matching controls were also compared.[19] We filtered the results to identify genes that were significantly deregulated in *DOCK6* KO clones and AOS patient cells.[19] We found that the mRNA of the small ubiquitin-like modifier Interferon Stimulated Gene 15 (*ISG15*) was downregulated 4-fold in both *DOCK6* KO and AOS patient fibroblast cells. Consistent with the expression profiling, ISG15 protein levels were lower in both *DOCK6* KO clonal HeLa cell line models than in wild type controls.[19]

Expression of *ISG15* is strongly induced by Type I α and β interferons (IFNs). ISG15 resides within cells in either a free form or conjugated to its target proteins. ISG15 is conjugated to its substrates via E1, E2 and E3 enzymes that function similar to the protein ubiquitination pathway.[30] ISGylation has been extensively studied in the context of innate cell immunity and immune diseases. It has been suggested that newly synthesized proteins become ISGylated by the E3 enzyme and subsequently degraded by the proteasome to combat viral infections. This mechanism would destroy virus-encoded proteins and so provide some level of protection from infection.[31] However, recent data suggest that ISG15 modulates the function of non-infected cells independently of its suggested role in protein degradation.[32,33] Moreover, *ISG15* is also deregulated in some cancer types.[34,35] In breast cancer cells, ISG15 depletion or interference with ISG15 conjugation to target proteins, counteracts hallmark cancer phenotypes such as the disruption of F-actin cytoskeleton, the reduction in the number of focal adhesions and the enhancement of cell motility that leads to metastasis.[32,33] These observations suggest a previously unappreciated link between the actin cytoskeleton and ISG15. How ISG15 is regulated in cancer cells and how it affects the actin cytoskeleton is presently unclear.

We found that ISG15 protein levels decreased gradually following DOCK6 depletion to reach their lowest levels after 96 hours, at which point cells starts to adapt to DOCK6 loss.[19] Consistently, double knockdown of DOCK6 and ISG15 was able to accelerate adaptation to DOCK6 function loss by buffering the phenotypes arising from DOCK6 depletion after standard 48 h of depletion. Importantly, overexpression of *ISG15* in *DOCK6* KO cells was able to reverse adaptation.[19] Thus, a decrease in ISG15 levels suppresses the loss of DOCK6 function.

The actin polymerization state at any given time can control gene transcription through the activities of Serum Response Factor (SRF) and myocardin-related transcription factors (MRTFs).[36] SRF binds to a specific sequence called the CArG box motif to regulate the transcription of its target genes.[36] We have identified a CArG box motif in the promoter region of *ISG15* and confirmed the binding of SRF/MRTF-A transcription machinery via chromosomal immunoprecipitation (ChIP) experiments.[19] MRTF-A is a co-activator of SRF which binds to SRF to activate it and increased levels of globular actin (G-actin) sequester MRTF-A in the cytoplasm leading to a decrease in SRF transcription activity.[36] We found that the increased levels of free G-actin in *DOCK6* KO cells bound to the SRF activator MRTF-A to retain it in the cytoplasm.[19] This retention explains the decreased *ISG15* expression in *DOCK6* KO cells and suggests a G-actin and MRTF-A/SRF dependent feedback loop that regulates ISG15 amounts at the level of transcription.[19]

Such clear impacts of ISG15 upon actin function begged the question as to the identity of the molecular target of ISGylation. We identified IQ motif Containing GTPase Activating Protein 1 (IQGAP1) as the major target of ISG15 conjugation system in this cell-intrinsic adaptation to sustained DOCK6 activity loss.[19] Mass spec experiments have shown that IQGAP1 was subject to ISGylation.[19,31,37] IQGAP1 is a scaffold protein that regulates the actin cytoskeleton and is known to stabilize active CDC42 and RAC1.[38-41] We showed that decreased ISGylation of IQGAP1 increased its binding affinity for

the active forms of CDC42 and RAC1. How IQGAP1 regulates the activities of CDC42 and RAC1 in dependence of ISG15 is presently unclear. The reduced affinity of CDC42-GTP and RAC1-GTP to ISG15-modified IQGAP1 may make these GTPases more accessible to inactivating GAPs. Alternatively, ISGylation may regulate the scaffolding function of IQGAP1, for example, releasing and activating a GEF or recruit and thereby inhibiting a GAP upon de-ISGylation of IQGAP1. This would activate CDC42 and RAC1 enabling cells to suppress the interphase and mitotic phenotypes arising from the absence of DOCK6.[19]

In this study we showed that DOCK6 co-localizes with ER during interphase and mitosis (Fig. 1). Given the described functions of DOCK6 as a CDC42/RAC1 dual specific GEF, its ER localization is interesting[19] (Fig. 3, adapted from refs. 42, 43). The cage like enclosure of the mitotic chromatin by the ER-associated DOCK6 may ensure maximal exposure of kinetochores to active GTP-bound CDC42. ER localization of DOCK6 in interphase could indicate an additional role of DOCK6 related to membrane trafficking. For example, CDC42 was shown to mediate membrane trafficking at Golgi/ER interface and DOCK6 GEF activity on CDC42 might be required for this CDC42 function[44]

Down-regulation of the ubiquitin-like modifier ISG15 is responsible for the suppression of the interphase and mitotic defects upon prolonged DOCK6 depletion (Fig. 3). *ISG15* transcription was regulated by the recruitment of MRTF-A/SRF transcription factor machinery to a CArG motif in the ISG15 promoter. Upon *DOCK6* disruption, a G-actin-MRTF-A/SRF-ISG15 feedback loop adapts the activity of mis-regulated RAC1/RHOA and CDC42 via IQGAP. We propose that this loop might be suppressing phenotypes in AOS patients and other disorders that are based on RAC1 and CDC42 deregulation. These findings open new possibilities for the treatment of diseases that affect actin biogenesis. Besides the ISG15-IQGAP1-RAC1 branch that we have described here, ISGylation probably has additional roles in regulating the actin cytoskeleton and cell motility since it has candidate target proteins related to the actin cytoskeleton, cell adhesion and motility such as CFL1 (Cofilin), PFN1 (Profilin-1), ACTN4 (Actinin α-4), FLNA/B (Filamin A/B), WDR-1 (WD-Repeat Protein-1) and VIL2 (Vilin-2/Ezrin).[19,37]

It is known that RNAi and CRISPR/Cas9 based gene inactivation approaches frequently lead to different phenotypes.[45] Here we have described how cell-intrinsic adaptation compensates for the loss of gene function in a manner that is dependent upon the duration of the depletion yet independent of the means by which the gene inactivation is invoked. It seems likely that prolonged gene inactivation is more representative of disease phenotypes, while acute inactivation reveals the molecular function in a given equilibrium. Thus, both strategies reveal important aspects of the function of a gene product and defining both states will most clearly show the full range of options available to repair or compensate for gene defects and metabolic disorders and eliminate cancer.

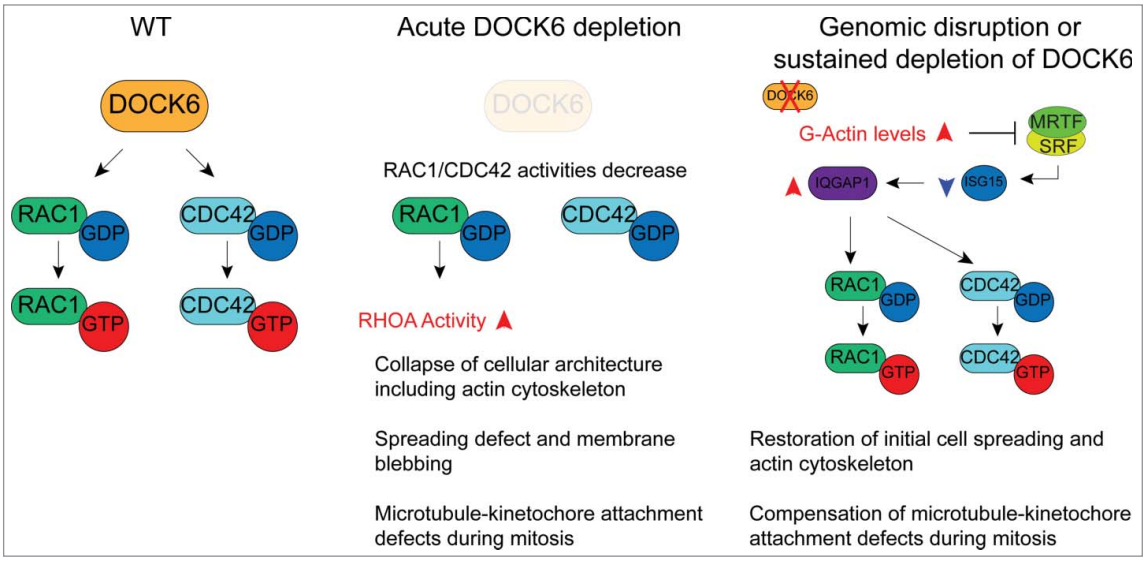

Figure 3. Model: Acute depletion vs. compensation to sustained DOCK6 loss and/or genomic disruption. See figure and text for description. Red arrowheads indicate increased activity or levels and the red arrowhead next to IQGAP1 indicates the increased binding of de-ISGylated IQGAP1 toward RAC1-GTP and CDC42-GTP.

Materials and methods

Mammalian cell culture and Flp-in T-Rex system

Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax (Gibco). Final media used contains 10% FBS, 1% P/S and 1% Na-Pyruvate. We thank Stephen Taylor (University of Manchester) for providing Flp-in T-Rex HeLa cell line which allows doxycycline induced ectopic gene expression and stable clones were created and maintained as described before.[46]

Cell synchronization and transfection conditions

Cells were seeded on fibronectin coated glass coverslips. Fibronectin coatings were made by incubating glass coverslips with 5 μg/cm^2 of fibronectin in PBS for 1 hour at room temperature (RT). For interphase co-localization experiments, cells were seeded on glass coverslips at 50% confluence and incubated overnight. Next day expression of *DOCK6* constructs was induced by addition of 1 μg/ml doxycycline and cells were transfected with RFP-KDEL plasmid with Lipofectamine 2000 according to manufacturer's protocol. RFP-KDEL construct was kindly provided by Marius Lemberg (University of Heidelberg). For mitosis experiments cells were incubated overnight with 2.5 mM of thymidine to induce an arrest in interphase and simultaneously transfected with *RFP-KDEL* construct and expression of *DOCK6* FL was induced with doxycycline. Next day, after thymidine removal cells were released into 100 ng/ml nocodazole containing media for 12 hours. After nocodazole washout, cells on the coverslips were fixed with 3% PFA every 10 min and prepared for immunofluorescence experiments.

Immunofluorescence microscopy

After washing the fixed cells with 1X PBS, cells were blocked with 10% FBS (Fetal Bovine Serum) for 1 hour. Cells were incubated with primary antibodies diluted in 3% BSA for 1 hour at room temperature. After washing cells 3 times with 1X PBS cells were incubated with Alexa-488, Alexa-555 or Alexa-647 conjugated secondary antibodies diluted in again 3% BSA containing 5 μg/ml Hoechst 33342 for 30 min at RT. After incubation with secondary antibody and Hoechst solution samples were washed 3 times with 1X PBS. Finally, the coverslips were mounted in Prolong Gold Antifade reagent (Molecular Probes: P36930). Samples were analyzed with DeltaVision Olympus IX71 microscope (Applied Precision) equipped with DAPI, FITC, TRITC, and Cy5 filters (Chroma Technology) and CoolSNAP HQ camera (Photometrics).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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