miR-34/449 control apical actin network formation during multiciliogenesis through small GTPase pathways

Benoît Chevalier¹,²,*, Anna Adamiok³,*, Olivier Mercey¹,²,*, Diego R. Revinski³, Laure-Emmanuelle Zaragosi¹,², Andrea Pasini³, Laurent Kodjabachian³, Pascal Barbry¹,² & Brice Marcet¹,²

Vertebrate multiciliated cells (MCCs) contribute to fluid propulsion in several biological processes. We previously showed that microRNAs of the miR-34/449 family trigger MCC differentiation by repressing cell cycle genes and the Notch pathway. Here, using human and Xenopus MCCs, we show that beyond this initial step, miR-34/449 later promote the assembly of an apical actin network, required for proper basal bodies anchoring. Identification of miR-34/449 targets related to small GTPase pathways led us to characterize R-Ras as a key regulator of this process. Protection of RRAS messenger RNA against miR-34/449 binding impairs actin cap formation and multiciliogenesis, despite a still active RhoA. We propose that miR-34/449 also promote relocalization of the actin binding protein Filamin-A, a known RRAS interactor, near basal bodies in MCCs. Our study illustrates the intricate role played by miR-34/449 in coordinating several steps of a complex differentiation programme by regulating distinct signalling pathways.
Multiciliated cells (MCCs), characterized by the presence of multiple motile cilia at their apical surface, have been described in many vertebrates\(^1,2\). Coordinated ciliary beating allows efficient fluid movement and is required for physiological processes such as elimination of mucus from the respiratory tract, circulation of the cerebrospinal fluid or migration of the embryo in the fallopian tubes\(^3\). The physiological importance of MCCs is highlighted by the ever growing number of human disorders associated with defects of the motile cilia\(^1,2,3\). Multiciliogenesis, which occurs during normal development and during regeneration of damaged tissues, can be studied in experimental setups, such as primary cultures of human airway epithelium\(^4\) and Xenopus embryonic epidermis\(^5\). Several characteristic steps are observed as follows: (i) exit from the cell cycle of MCC precursors, (ii) massive postmitotic multiplication of centrioles (centriologenesis), (iii) reorganization of the apical actin cytoskeleton into a dense cortical meshwork of actin, (iv) migration of the newly synthesized centrioles towards the apical pole of the cell, where they anchor to the apical membrane and mature into ciliary organizing centres known as basal bodies, and (v) elongation of one cilium from each basal body\(^6\)–\(^10\). Several key regulators of multiciliogenesis have been identified, such as Notch and bone morphogenetic protein (BMP) pathways\(^11\)–\(^14\), the transcription factors FOXJ1, MB2 and RXFs (regulatory factor X)\(^15\)–\(^17\) and the geminin-related nuclear protein Multicilin\(^25\). During multiciliogenesis, the reorganization of the apical actin cytoskeleton is controlled by several factors including FOXJ1, Multicilin, the ERK7 mitogen-activated protein kinase and small GTPases such as RhoA\(^14\),\(^19\),\(^20\),\(^25\)–\(^28\). Following FOXJ1- and RhoA-pathway-dependent phosphorylation, proteins of the ezrin-radixin-moesin (ERM) family, which link actin to the cell membrane, can interact with cortical actin\(^29\),\(^30\). The subcellular localization of ezrin and its interacting protein EB5 in the apical membrane of airway MCCs also appears to be mediated by a FOXJ1-dependent mechanism\(^14\),\(^19\),\(^31\),\(^32\). Focal adhesion proteins are also required for the interaction between basal bodies and apical actin network during multiciliogenesis\(^33\). The action of small GTPases on actin cytoskeletal dynamics is regulated by a complex network of interactions with additional GTPases, such as the Ras family member R-Ras\(^34\)–\(^39\), and other regulatory factors including guanine nucleotide exchange factors, GTPase-activating proteins (GAPs), GDP-dissociation inhibitors (GDIs)\(^40\),\(^41\) and microRNAs (miRNAs)\(^42\). Recent work has also highlighted the importance of interactions between the Ras GTPase and the planar cell polarity pathway in controlling the assembly of apical actin filaments, as well as the docking and planar polarization of the basal bodies in MCCs\(^43\),\(^44\).

miRNAs or miRs are a class of small single-stranded and non-coding regulatory RNAs that control many biological processes by limiting the stability and the translation of their target mRNAs\(^45\),\(^46\). Abnormal miRNA activity has been associated with a wide variety of human pathologies including airway diseases\(^47\). We have previously demonstrated that the miR-34/449 family is important for the initiation of human and Xenopus MCC differentiation. Members of this family share high sequence homology and miR-449a/b/c, which are located on the same genomic locus as Multicilin, were identified as the most strongly induced miRNA species in human and Xenopus during MCC differentiation. We showed in these two species that miR-34/449 promote cell cycle exit and entry into differentiation by repressing several components of the cell cycle control machinery and of the Notch signalling pathway\(^5\). Their inactivation was sufficient to block centriole amplification and multiple motile cilia formation\(^5\). Two recent studies confirmed our findings by showing that miR-34/449-deficient mice exhibited impaired multiciliogenesis\(^48\),\(^49\).

Song et al.\(^48\) also showed that the centriolar protein Cp110 is an additional important miR-34/449 target that must be repressed to allow the maturation of basal bodies. Thus, beyond their initial effect on cell cycle exit and entry into differentiation, miR-34/449 probably regulate later steps of the complex multiciliogenesis process. As the development of functional motile cilia appears exquisitely sensitive to the reorganization of the apical actin cytoskeleton, we reasoned that miR-34/449 may also regulate one or more molecules associated with actin dynamics or small GTPase pathways. Here we show that miR-34/449 indeed contribute to the establishment of the apical actin cytoskeleton, via a mechanism involving the direct repression of the small GTPase R-Ras. This further establishes miR-34/449 as a central control system of multiciliogenesis acting at several distinct levels of this complex physiological process.

Results and Discussion

The apical actin network of human and Xenopus MCCs. Apical actin cytoskeleton formation was examined at several time points during differentiation of primary cultures of human airway epithelial cells (HAECs) grown at an air–liquid interface (ALI) and in Xenopus embryonic epidermis\(^9\). Formation of the apical meshwork of filamentous actin (F-actin) was monitored directly by staining with fluorescent phalloidin and indirectly by staining for ezrin or phospho-ERM. In human and Xenopus, the acetylated tubulin-positive MCCs displayed a strong enrichment of apical F-actin (human: Supplementary Fig. 1a–d; Xenopus: Supplementary Fig. 1f). Basal bodies, which are positive for γ-tubulin labelling, are embedded within an apical F-actin and ezrin meshwork (Supplementary Fig. 1b). Reorganization of actin filaments involves cofilin, a ubiquitous G-actin-binding factor\(^50\). As unphosphorylated cofilin depolymerizes actin filaments, cofilin phosphorylation appears essential for cytoskeletal reorganization\(^50\). Phosphorylation of cofilin-1 also contributes to actin network stabilization and formation of focal adhesions\(^51\). Focal adhesion proteins indeed participate to ciliary adhesion complexes in MCCs and allow interactions between basal bodies and the apical actin network\(^33\). In HAECs, the levels of phosphorylated cofilin-1 and ezrin increased during MCC differentiation (Supplementary Fig. 1e). We noticed a parallel increase in the expression of the ERM-binding protein EB50 (Supplementary Fig. 1e), an adapter protein required for the maintenance of active ERM proteins at the apical membrane of polarized epithelia\(^14\),\(^32\). Interestingly, we also observed a punctate labelling of phosphorylated cofilin-1, which was localized sub-apically close to basal bodies in MCCs, but was absent from non-ciliated cells (Supplementary Fig. 1c,d).

miR-34/449 control apical actin network assembly in MCCs. We examined whether the miR-34/449 family can control the formation of the apical actin network, a prerequisite for basal body anchoring and cilium elongation. To invalidate miR-449 or miR-34-34/b activity in human, we transfected HAECs with a cholesterol-conjugated antagonomiR directed against miR-449 (Antago-449) or against miR-34 (Antago-34) and assessed MCC differentiation. Antago-449 as well as antago-34 strongly blocked miR-449a/b, whereas antago-34 blocked miR-34a/b/c more efficiently than antago-449 (Supplementary Fig. 2b). Antago-449 and antago-34 blocked to the same extent the formation of MCCs, and no additive effect was observed (Supplementary Fig. 2c). The huge induction of miR-449 expression at early ciliogenesis (stage EC) suggests that early effects were mainly mediated through miR-449, without excluding a later role for miR-34 (Supplementary Fig. 2a). This could also explain the lack of...
compensation of miR-34 in our antago-449 conditions, which do not alter the expression of miR-34. In HAECS, miR-449 silencing caused a decrease of 51% ± 3.5 in the number of acetylated tubulin-positive cells and of 33% ± 7 in the number of ezrin-positive cells (Fig. 1b). In *Xenopus* epidermis, miR-449a and miR-34b were both specifically expressed in MCCs, and their levels in tubulin-positive cells (Supplementary Fig. 2f), suggesting that 449-MOs into the prospective epidermis at the eight-cell stage. In *Xenopus*, miR-34b was detected in MCCs (Supplementary Fig. 2d–f), and in situ hybridization (ISH) experiments also revealed that 449-MOs not only blocked the expression of miR-449 but also blocked the expression of miR-34b (Supplementary Fig. 2f). MiR-449 knockdown suppressed multiciliogenesis and apical actin web formation in both HAECS (Fig. 1a, b) and *Xenopus* embryonic epidermis (Fig. 2a, b). In embryos injected with 449-MOs, the number of acetylated tubulin-positive cells went down to 18% ± 13 of the control and the number of apical actin cap-positive cells decreased to 9% ± 8 of the control (Fig. 2a, b). These results establish that the miR-34/449 family interferes with MCC apical meshwork formation in both models. As miR-34 and miR-449 miRNAs share the same targets, we only used miR-449 in the rest of this study. The impact of miR-449 on the actin cytoskeleton was further investigated by monitoring the formation of focal adhesion and stress fibres, which are thick and relatively stable actin filaments involved in cell adhesion and morphogenesis. We found that in proliferating A549 cells, a human lung cell line devoid of miR-449 and miR-34b/c, miR-449 overexpression increased actin stress fibres and focal adhesion formation (Fig. 1c, d). In addition, western blot analysis revealed increased ERK phosphorylation following miR-449 transfection in proliferating HAECS (Fig. 1e, c), consistent with the regulatory role of phospho-ERM during actin cytokinetic dynamics. This, our data show that the miR-34/449 family clearly contributes to actin cytoskeleton remodelling in several independent models. Next, we addressed the precise mode of action of miR-34/449 in the construction of the apical actin network in MCCs.

Mutual repression between miR-449 and the Notch pathway. As the miR-34/449 family represses the Notch pathway during MCC differentiation, we assessed the contribution of the Notch signal to the actin web reorganization. We treated proliferating HAECS or human lung A549 cells (which both are devoid of endogenous miR-449) either with miR-449 or with N-[N-(3,5-Difluorophenacyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor that blocks Notch activation. As expected, both ectopic expression of miR-449 in proliferating HAECS and DAPT repressed the expression of the Notch target gene HES1 (Fig. 1g). In proliferating A549 cells, Notch inhibition with DAPT alone had neither impact on the formation of actin stress fibres and focal adhesions (Fig. 1c, d) nor on ERK phosphorylation in proliferating HAECS (Fig. 1e). This suggests that miR-449 cause a rise of levels of phosphorylated ERM independently of Notch repression.

We observed that preventing the binding of miR-34/449 on Notch1 (PO-Notch1 in HAECS, Fig. 1a, b) or on the Notch ligand DLL1 (PO-DLL1 in *Xenopus*, Fig. 2c–f) with protector oligo-nucleotides coordinately blocked multiciliogenesis and apical actin network formation. This is consistent with the need for an early repression of the Notch pathway by miR-34/449, to allow MCC differentiation. Of note, in *Xenopus*, the percentage of cells exhibiting defective apical actin meshwork was higher in miR-34/449 morphants (Fig. 2b) than in PO-DLL1 morphants (Fig. 2d), suggesting that miR-34/449 may affect additional targets. We also noticed a reduction of miR-449 levels when preventing miR-34/449 binding on Notch1 in differentiating HAECS (Fig. 1f) and on DLL1 in frog epidermis (Fig. 2f). Conversely, miR-449 expression was increased after treatment of differentiated HAECS with DAPT (Fig. 1f). Altogether, these data reveal the existence of a double-negative feedback loop between miR-449 and the Notch pathway. We hypothesize that once this loop is locked in a state of high miR-449 expression, interactions of miR-449 with additional targets expressed at subsequent steps of multiciliogenesis remain possible (see Fig. 8d). According to a recent work, CP110 would represent one such target. Its repression by miR-34/449 appears to affect centriole maturation, but not apical actin network assembly. We thus looked for other possible targets of miR-34/449 that would be directly related to actin dynamics.

miR-34/449 targets components of the small GTPase pathways. Several small GTPase proteins such as RhoA or Rac1 act as key regulators of multiciliogenesis. As miR-34 and miR-449 miRNAs share the same targets, we only used miR-449 in the rest of this study. The modulation of RhoA activity by miR-34/449 may play a role in the control of apical actin polymerization, other miR-34/449 targets contribute to the profound disruption of the actin cap observed after miR-34/449 inactivation.

In a bid to identify such additional factors, we applied several miRNA target prediction tools to identify putative miR-34/449 targets among the small GTPase pathways. Specifically, we looked for relevant miR-34/449 mRNA targets that were repressed during MCC differentiation and after overexpression of miR-34/449 in proliferating HAECS (Gene Expression Omnibus
Figure 1 | MiR-449 affects actin network remodelling and multiciliogenesis in HAECs. (a-c) Effect of a treatment by control antagomiR (CTR-Neg), anti-miR-449a/b (Antago-449) and miR-449::Notch1 protector (PO-Notch1) on differentiating HAECs. (a) Staining for F-actin (1,5,9), ezrin (2,6,10) and acetylated tubulin (3,7,11), at LC stage. (b) The histogram indicates the average percentage of MCCs (in magenta) and apical ezrin-positive (in green) cell number relative to control (means ± s.d. from nine and three donors for MCC and ezrin quantifications, respectively. ***P < 0.001, **P < 0.01; Student’s t-test). (c) Immunostaining of focal adhesions protein Paxillin (in green), F-actin (in red) and nuclei (in blue) in A549 epithelial cells transfected for 72 h with control miRNA (miR-Neg), miR-449a or miR-Neg plus 10 μM DAPT. (d) Ratio of focal adhesion number per cell, normalized to control (n = 5 fields in three independent experiments; ***P < 0.001; Student’s t-test). (e) Effect of miR-449 overexpression and DAPT (10 μM) on ERM phosphorylation in proliferating HAECs. Phosphorylated protein levels were normalized with non-phosphorylated ERM and with an antibody against HSP60 as a loading control. Normalized fold changes are indicated on the corresponding bands. Experiments were representative of three donors. (f) Effect of PO-Notch1 and DAPT (10 μM) in differentiating HAECs at LC stage on miR-449 expression, normalized with RNU44. (g) Real-time RT–PCR of HES1 transcripts in control, DAPT (10 μM, 48 h)-treated or miR-449-overexpressing proliferating HAECs. Transcript levels of HES1 were normalized against UBC transcript as an internal control. (f,g) Data represent the mean and s.d. of three independent experiments (***P < 0.001, **P < 0.01; Student’s t-test).
Figure 2 | MiR-449 affects actin network remodelling and multiciliogenesis in Xenopus epidermis. (a–d) The epidermis precursor blastomeres (eight-cell stage Xenopus embryos) were injected with negative control morpholinos (CTR-Neg) (a,c), with morpholinos against miR-449 (449-MOs) (a,b) or with protector morpholinos of Dll1 (PO-Dll1) (c,d). Staining at stage 25 for F-Actin (in red, a1,5 and c1,5) and motile cilia (Ac. Tub. in magenta, a3,7 and c3,7). Injected cells were detected by the expression of a synthetic mRNA coding for membrane-bound GFP (GFP-CAAX in green, a2,6 and c2,6). (b) The histogram indicates the percentage of GFP-CAAX-positive injected cells that develop motile cilia or apical actin cap in controls (Stage 24 + 25: n = 5 fields/583 injected cells) and in miR-449 morphants (Stage 24 + 25: n = 8 fields/625 injected cells; P-value $\alpha_{24 + 25} = 0.0087$; Mann-Whitney test with two-tailed P-value). (d) Percentage of injected cells positive for GFP fluorescence with normal or defective actin cap in control (n = 30 fields per 1,345 injected cells) versus PO-Dll1 morphants (n = 32 fields per 1,268 injected cells; P-value (normal versus defective in control) $\alpha_{24 + 25} < 0.0001$, P value (normal versus defective in PO-Dll1-injected $\alpha_{24 + 25} = 0.0033$; Mann-Whitney test with two-tailed P-value). (e) Effect of protecting the Dll1 mRNA from the interaction with miR-449 (PO-Dll1) in Xenopus epidermis on Dll1 expression (normalized with ornithine decarboxylase (ODC)). (f) Effect of PO-Dll1 in Xenopus epidermis on miR-449 expression, normalized with U6. Data are means ± s.d. of two independent experiments.
Figure 3 | miR-449 controls small GTPase pathways during MCC differentiation. (a) Left (Prolif. HAECs): RhoA activity in proliferating HAECs transfected for 72 h with miR-Neg, miR-449a and/or incubated with DAPT (10 μM) or a Rho activator (calpeptin, 1 U ml⁻¹, 2 h). Right (Diff. HAECs): RhoA activity in differentiating HAECs at Po stage treated for 72 h with antago-Neg, antago-449 or DAPT (10 μM). RhoA activity is expressed as a percentage relative to control. Data are mean ± s.d. from at least three independent experiments (⁎P < 0.05, ⁎⁎P < 0.01 and ⁎⁎⁎P < 0.001; Student’s t-test). (b) RhoA activity in Xenopus embryos at stage 25, assessed by measuring the fluorescence of rhod integrin rGBD-GFP, an active RhoA sensor (green, upper panels). Injected cells are identified by the membrane-bound RFP (mRFP in red, lower panels). Cells were either untreated (CTR-Neg, b1, 2, 5, 6) or with Notch intracellular domain NICD (b3, 4). Motile cilia’s staining is in magenta (lower panels). (c) The histogram indicates the percentage of rGBD-GFP-positive cells stained for α-tubulin mRNA (α-Tub. in black) or acetylated tubulin (Ac. Tub. in magenta) in miR-449 morphants (449-MOs, n = 68) relative to negative control (n = 110). Data are mean ± s.e.m. (⁎⁎P < 0.005, ns, not significant, one-way analysis of variance with Dunnett’s test).

(GEO) data set GSE22147). This survey led us to identify several candidates related to small GTPase signalling and actin cytoskeleton remodelling: (1) ARHGAP1, a member of the Rho-GAP family; (2) ARHGDIB, also called Rho-GDI2 (ref. 40); (3) DAAM1, the diaphanous-related formin Dishevelled-associated activator of morphogenesis; (4) NDRG1, an iron-regulated metastasis suppressor; (5) R-Ras, a member of the superfamily of small GTPases, related to Ras.

By looking more precisely at the three major families of regulators of small GTPases expressed during HAECs differentiation or after miR-34/449 transfection, we detected 23 distinct ARHGAPs, including ARHGAP1; we also detected 22 distinct ARHGDEF transcripts but none of them were predicted as direct miR-34/449 targets; regarding the three known mammalian ARHGD1 transcripts, only ARHGDIB was further analysed, as ARHGDA expression levels did not change during HAECs differentiation or after miR-34/449 transfection and ARHGDIG was not detected (Supplementary Fig. 3b, see also GEO GSE22147).

The transcripts ARHGAP1, ARHGDB, DAAM1, NDRG1 and RRAS are all modulated during HAEC differentiation (Fig. 4a, see also GEO GSE22147). Figure 4a shows that the transcript level of ARHGAP1, DAAM1 and NDRG1 decreased at late ciliogenesis (LC). The expression of ARHGDB transcript slightly decreased at the onset of differentiation (that is, polarization step, Po) but rose again during the phase of multiciogenesis (Fig. 4a). The RRAS transcript level decreased throughout the whole time course of MCC differentiation (Fig. 4a). In proliferating HAECs, miR-449 overexpression strongly reduced the transcript levels of ARHGDB, ARHGAP1 and RRAS, whereas the expression of DAAM1 and NDRG1 transcripts was slightly decreased (Fig. 4b). These putative targets were further investigated using a dual luciferase reporter assay in HEK293 cells. MiR-449a and miR-449b reduced the relative luciferase activity of chimeric constructs containing the wild-type 3′-untranslated regions (3′-UTRs) of ARHGAP1, ARHGDB, NDRG1 and RRAS, but not of DAAM1 3′-UTR (Fig. 4c). Next, we focused on the three most significantly regulated miR-34/449 targets ARHGAP1, ARHGDB and RRAS. MiR-449-mediated silencing of either ARHGAP1, ARHGDB or RRAS was respectively abolished when miR-34/449-predicted binding sites were mutated (Fig. 4c). Among the four miR-449-binding sites in the 3′-UTR of RRAS, the strongest effect was observed for the most 3′-site, which also corresponds to the unique conserved site between human and...
**Figure 4** | ARHGAP1, ARHGDIB and RRAS are targeted by miR-449 in HAECs. (a,b) Transcript expression levels of ARHGAP1, ARHGDIB, DAAM1, NDRG1 or RRAS were analysed using real-time RT-PCR during HAEC differentiation (a) or following miR-449a overexpression (48 h) in proliferating HAECs (b), and normalized with UBC transcript as an internal control. (c) Specific interaction between miR-449a/b and the 3’-UTRs of ARHGAP1, ARHGDIB, DAAM1, NDRG1 and RRAS mRNAs was assessed using luciferase reporter assay on constructs carrying either the wild-type (wt) or mutants (mut.) 3’-UTR-binding sites for miR-449. Values were normalized with the internal *Renilla* luciferase control. (d) Detection of ARHGAP1, ARHGDIB and R-Ras proteins after miR-449 overexpression in proliferating HAECs for 72 h. HSP60 is used as an internal control. Quantification of protein levels are indicated above each corresponding band. All data are means ± s.d. of at least three independent experiments (***/P < 0.001, ***P < 0.01 and *P < 0.05; Student’s t-test).
context of multiciliogenesis. As the pattern of expression of both arhgap1 and arhgdib in Xenopus epidermis was not consistent with an association to MCC precursors, arhgap1 and arhgdib were not further analysed in Xenopus.

We designed target protection assays in which cholesterol-conjugated modified oligonucleotides were transfected in differentiating HAECs to compete with the binding of miR-34/449 on the site identified within the human 3′-UTR of ARHGDIB.
miR-34/449 control apical actin assembly by repressing R-Ras.

We used target protection assays (cholesterol-conjugated modified oligonucleotides in HAECs or morpholino oligonucleotides in frog epidermis) to compete with the binding of miR-34/449 on sites identified within the human and Xenopus 3′-UTRs of RRAS mRNA. In human cells, the RRAS protector oligonucleotide (PO-RRAS) strategy effectively blocked the action of ectopic miR-449, as evidenced on RRAS 3′-UTR in luciferase assays (Supplementary Fig. 4b). In addition, miR-34/449 knockdown or protection of RRAS mRNA from miR-34/449 in frog epidermis led to an increase in rras transcript levels (Supplementary Fig. 4c).

We checked that in both species the expression of RRAS2, an RRAS-related gene, did not interfere with R-Ras signalling. RRAS2 expression remained at a very low level during MCC differentiation and in response to miR-449 overexpression, and was not altered by miR-34/449 knockdown or PO-RRAS protection (HAECs, see GEO GSE2147; Xenopus, Supplementary Fig. 4c). In both models, the PO-RRAS strategy was also able to increase endogenous R-Ras protein level (Supplementary Fig. 4d,e).

Collectively, these assays unambiguously establish that RRAS transcripts were specifically targeted by miR-34/449 in human and Xenopus MCCs.

In human (Fig. 6a,b) and in Xenopus (Fig. 7a,d), protection of the RRAS transcript from miR-34/449 binding led to a strong reduction in apical actin meshwork and motile cilia formation. Silencing RRAS in human at an early step of HAEC differentiation also strongly affected apical actin meshwork formation and blocked multiciliogenesis (Fig. 6b,c). These results strongly suggest that R-Ras plays a key role in maturation of MCCs, in which its level of expression has to be tightly fine-tuned, to allow apical actin cap formation and multiciliogenesis. In frog epidermis, both apical and sub-apical phalloidin staining were altered in PO-rras-injected embryos (Fig. 7a,b). In contrast, F-actin staining remained detectable at cellular junctions in both models. Importantly, actin cap formation and multiciliogenesis were rescued in Xenopus epidermis when a morpholino designed to block R-Ras translation (MO-ATG-rras) was co-injected with PO-rras (Fig. 7a,d). At the dose used for this assay, injection of MO-ATG-rras alone had no significant effect on either apical actin meshwork formation or multiciliogenesis (Fig. 7a–d), suggesting that rras expression may be already repressed by the presence of endogenous miR-34/449 in those MO-ATG-rras maturing MCCs.

Our data unambiguously indicate that the repression of RRAS at a late step of MCC differentiation by miR-34/449 is required for apical actin network assembly and multiciliogenesis in human (Fig. 6) as well as in frog (Fig. 7). Considering that R-Ras activity can be increased by Notch pathway activation, as previously observed in another cellular model, and considering the early repression of Notch signalling by miR-34/449 during vertebrate multiciliogenesis, miR-34/449 is therefore able to control R-Ras function at two distinct levels: (1) directly, via the inhibition of RRAS expression, and (2) indirectly, via the inhibition of R-Ras activity through the repression of the Notch pathway.

As observed for miR-449 morphants with rhodokin staining in Xenopus epidermis (Fig. 3b), PO-rras-injected MCCs displayed reduced actin cap and motile cilia staining, but maintained RhoA activation at the apical surface (Fig. 7c,e). This suggests that RRAS may interact with downstream effectors of RhoA during actin cap formation. In particular, previous works described an interaction between R-Ras and Filamin A (FLNA), a non-muscle F-actin cross-linking protein involved in epithelial cell shape, actin cytoskeleton remodelling and primary cilium formation. FLNA can interact with the RhoA and Shroom3 signalling pathways, and has also been involved in ciliogenesis and basal body positioning through its interaction with Meckelin.
We detected in control fully differentiated HAECs (Fig. 8a) FLNA in MCCs near apically docked basal bodies. By contrast, it was more homogeneously distributed in non-ciliated cells. The same apical enrichment of FLNA was observed in Xenopus MCCs (Fig. 8c). We finally noticed a miR-449-dependent subcellular redistribution of FLNA, after quantifying R-Ras and FLNA protein levels in different cellular fractions of proliferating HAECs that overexpress or not miR-449. In the absence of miR-449, R-Ras and FLNA were enriched in the membrane fraction (Fig. 8b). Following miR-449 overexpression, R-Ras disappeared from the membrane fraction, while FLNA was redistributed from the membrane to the cytoskeletal fraction (Fig. 8b). In a tentative model (Fig. 8d), we propose that the silencing of R-Ras by miR-34/449 in MCCs affects the interaction between R-Ras and FLNA, and favours a redistribution of FLNA in a cytoskeletal components involved into the anchoring of basal...

**Figure 7 | The direct repression of RRAS by miR-449 affects multiciliogenesis and apical actin cap formation in Xenopus.** (a,b) Eight-cell stage Xenopus embryos were injected in the epidermis precursor blastomeres with a mixture of synthetic mRNA coding for GFP-CAAX to label the injected cells (in green, a2,6,10,14) and negative control MO (CTR-Neg, a1–4, b1,2), or a morpholino protecting ras against binding by miR-449 (PO-ras, a5–8, b3,4) or a morpholino blocking the translation of ras (MO-ATG-ras, a9–12), or a combination of PO-ras and MO-ATG-ras (a13–16). MCCs are stained with an anti-acetylated tubulin antibody (in magenta, a3,7,11,15 and c1,2). F-actin with phalloidin (red, a1,5,9,13 and b1–4). (b) The impact of PO-ras in MCCs on apical (upper b5) and sub-apical actin (lower b6) was observed with F-actin staining. (c) In independent experiments, we used rGBD-GFP to examine RhoA activity in Xenopus MCCs (c1–4). Eight-cell stage Xenopus embryos were injected in the epidermis precursor blastomeres with a reporter of RhoA activity (rGBD-GFP in green, c1–4) and with negative control MO (CTR-Neg, c1–2) or with PO-ras (c3–4). MCCs are stained in magenta (c2,4). Protecting the ras mRNA from interaction with miR-449 results in defects in actin cap formation (F-Actin, a5 and b3,4) together with a loss of MCCs (Ac. Tub., a7,c4) without affecting apical RhoA activity (rGBD-GFP, c1,3). This phenotype is rescued when the translation of the protected ras mRNA is blocked by coinjection of MO-ATG-ras (a13–16). (d) The histogram indicates the percentage of injected cells (positive for mGFP) that develop proper apical actin cap (in red) and motile cilia (in magenta) in Xenopus epidermis at stage 25. CTR-Neg, n = 10 embryos per 413 injected cells; PO-ras, n = 8 embryos per 350 injected cells; MO-ATG-ras, n = 8 embryos per 290 injected cells; MO-PO-ras + MO-ATG-ras, n = 9 embryos per 395 injected cells. (e) The histogram indicates the percentage of rGBD-GFP-positive cells stained for α-tubulin (α-Tub. in black) or acetylated tubulin (Ac. Tub. in magenta) in PO-ras morphants (PO-ras, n = 150) in comparison with the negative control (CTR-Neg, n = 110). (**P < 0.005; ns, no significant; one-way-analysis of variance with Dunnett’s test). Data are mean ± s.e.m.
bodies. This mechanism would contribute to apical actin reorganization and basal body docking in MCCs.

In conclusion, our data further document how the miR-34/449 family can participate to multiciliogenesis through the repression of several important targets. MiR-34/449 may initially downregulate the expression of several cell cycle-regulated genes and members of the Notch pathway to promote entry into differentiation. Interestingly, miR-449 appears to be negatively regulated by Notch activity, supporting the existence of a double-negative feedback loop. At later time points of differentiation, this regulatory loop drives the accumulation of miR-449 at sufficient levels to downregulate additional targets implicated in more downstream events, such as CP110, involved in basal body maturation\(^{48}\), and R-Ras, shown here to be important for actin network assembly. From a wider point of view, this study illustrates well how a single miRNA family can contribute to complex cellular processes through its action on multiple targets belonging to different signalling pathways.

**Methods**

**Subjects/tissue samples.** Inferior turbinates or nasal polyps were from patients who underwent surgical intervention for nasal obstruction or septoplasty (kindly provided by Professor Castillo, Pasteur Hospital, Nice, France, or by Epithelix Sàrl, Geneva, Switzerland). The use of human tissues was authorized by the bioethical law 94–654 of the French Public Health Code after written consent from the patients.

**Isolation and culture of HAECs.** Primary HAECs were dissociated and seeded on porous polyester membranes (5 \( \times \) 10⁴ cells per membrane), in cell culture inserts (Corning-well-clear: 0.33 cm², 0.4 μm pores; Corning, Acton, MA) coated with human placenta collagen (0.2 mg ml\(^{-1}\); Sigma-Aldrich). HAECs were cultured in liquid–liquid conditions in the proliferation medium until confluency (5 days). Then, the culture medium was removed from the upper compartment and the airway epithelium was allowed to differentiate by using the differentiation medium consisting of 1:1 DMEM (Invitrogen, Gibco) and bronchial epithelial basal medium (Lonza) with the Clonetics supplements for human Epidermal Growth Factor (hEGF) (0.5 ng ml\(^{-1}\)), epinephrine (5 g ml\(^{-1}\)), bovine pituitary extract (BPE) (Lonza) and hEGF (0.5 ng ml\(^{-1}\)), epinephrine (5 g ml\(^{-1}\)), bovine pituitary extract (BPE)

**Figure 8** MiR-34/449 promote FLNA relocalization during MCC differentiation. (a) Apical layer of differentiated HAECs (LC stage) were stained for the basal bodies marker centrin-2 (in red) and the actin-binding protein filament-A (FLNA, in green). FLNA labelling was enriched in the apical layer near basal bodies (a1–3). Basal layer of differentiated HAECs (LC stage) were stained for FLNA (in green) and for nuclei with 4,6-diamindino-2-phenylindole (DAPI; in blue) (a4). (b) Modulation of protein levels of FLNA and R-Ras in total fraction (Total), membrane fraction (Membrane) and cytoskeletal fraction (Cytoskeleton) isolated from proliferating HAECs transfected for 72 h with miR-Neg or miR-449a as indicated each below corresponding band. Protein levels were normalized against HSP60 as an internal control for the total fraction and normalized fold changes are indicated above the corresponding bands. Data were representative of three independent experiments. (c) In the epidermis of stage 25 Xenopus embryos, FLNA labelling (in green, e1) is apically enriched in acetylated tubulin-positive MCCs (in magenta, c2,3). (d) Model illustrating the roles of miR-449 and other interconnected actors in MCC differentiation. Early cues required to trigger MCC differentiation involve the inhibition of BMP and Notch pathway. The expression of Multicilin (MCIDAS), CCNO and miR-449 is controlled by the Notch pathway activity. Notch repression is associated with the increase in MCIDAS, CCNO and miR-449 expression. Then, miR-449 miRNAs repress the Notch pathway inducing a double-negative feedback loop increasing miR-449 expression. MiR-449 inhibit cell cycle-related genes to stop proliferation and promote entry in differentiation. In addition, MCIDAS drives expression of centriole multiplication-related genes including CCNO, which then participates to centriole assembly and amplification. MCIDAS also contributes to increase FOXJ1 expression, which in turn controls ciliogenesis-related genes and apical actin remodelling through a RhoA-dependent mechanism. In parallel, miR-449 also control apical actin network remodelling by repressing R-Ras, promoting FLNA redistribution and modulating RhoA activity. Finally, miR-449 favour basal body maturation and anchoring by downregulating CP110. All these events are key prerequisites for axoneme elongation and motile cilia formation during MCC differentiation. Plain lines indicate direct interactions; dotted lines identify pathways that may or may not be direct.
(0.13 mg ml⁻¹), hydrocortisone (0.5 ml⁻¹), insulin (5 g ml⁻¹), tri-o-dithyronine (6.5 g ml⁻¹) and transferrin (0.5 ml⁻¹), supplemented with 200 U ml⁻¹ penicillin, 200 IU ml⁻¹ streptomycin and 0.1 ml⁻¹ reitnin acid (Sigma-Aldrich) in the basal compartment. We analysed HAEC differentiation at four time points where Pr, Po, EC and LC represent the proliferating step at day 0, the polarization step at day 7, the early multicilogenesis step at day 14 and the late multicilogenesis step at day 21, respectively. Day number corresponds to the number of days after setting up the cells at an ALI.

**Xenopus injections.** Eggs obtained from NASCO females were fertilized in vitro, dejellied in 2% cystein hydrochloride (pH 8.0) and cultured in modified Barth’s.

**Immunocytochemistry.** C. Chang, University of Alabama at Birmingham, USA) was linearized with Message mMachine kit (Life Technologies). pCS105/mGFP-CAAX (a gift from NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9386 | www.nature.com/naturecommunications

**Immunoblotting.** Cells, Thermo Scientific). Western blot experiments were repeated at least twice on separate injections and the qRT–PCR was performed in triplicate. The relative expression of **RRAS** was normalized to the expression of the housekeeping gene *ornithine decarboxylase*. The qRT–PCR **RRAS** primers are as follows: forward: 5’-gtactaaatggagagctgct-3’, reverse: 5’-gattgaacaaaggaacggaacttt-3’.

**MIR-449 silencing and target protection experiments.** Human, 3’-Cholesterol linked 2’-O-methyl mIR-449a/b or mIR-34/b-c antisense oligonucleotide (antagomiR, antago-449: 5’-cuaauauuucauaucuanuuu-3’, antago-34: 5’-gacgucaucagcaacagcuc-3’, miR-449b (full match) and miR-449b with one mismatch. The RRAS protector is a complementary reverse oligonucleotide targeting the conserved mIR-449 binding site of the human RRAS 3’-UTR. The antagomiR or protector negative control was the Clear-mIR-5’-cuaauauuucauaucuanuuu-3’, from Eurogentec. AntagomiR or antisense protector (100 μM) were pre-incubated with FCS for 30 min at room temperature. mIR-449a/antagomiR/FCS or protector/FCS mixture in 5’-auggcagagtacagcucaacgau-3’ and RRAS protector oligonucleotide (PO-RRAS: 5’-gugcaucagcaacagcuc-3’, from Eurogentec (Seraing, Belgique). Phosphorothioate bonds are indicated by subscript ‘s’. The miR-449 antagomiR targets Homo sapiens mIR-449a (full match) and miR-449b with one mismatch. The RRAS protector is a complementary reverse oligonucleotide targeting the conserved mIR-449 binding site of the human RRAS 3’-UTR. The antagomiR or protector negative control was the Clear-mIR-5’-cuaauauuucauaucuanuuu-3’, from Eurogentec. AntagomiR or antisense protector (100 μM) were pre-incubated with FCS for 30 min at room temperature. mIR-449a/antagomiR/FCS or protector/FCS mixture in 5’-auggcagagtacagcucaacgau-3’ and RRAS protector oligonucleotide (PO-RRAS: 5’-gugcaucagcaacagcuc-3’, from Eurogentec (Seraing, Belgique). Phosphorothioate bonds are indicated by subscript ‘s’. The miR-449 antagomiR targets Homo sapiens mIR-449a (full match) and miR-449b with one mismatch. The RRAS protector is a complementary reverse oligonucleotide targeting the conserved mIR-449 binding site of the human RRAS 3’-UTR. The antagomiR or protector negative control was the Clear-mIR-5’-cuaauauuucauaucuanuuu-3’, from Eurogentec. AntagomiR or antisense protector (100 μM) were pre-incubated with FCS for 30 min at room temperature. mIR-449a/antagomiR/FCS or protector/FCS mixture in 5’-auggcagagtacagcucaacgau-3’ and RRAS protector oligonucleotide (PO-RRAS: 5’-gugcaucagcaacagcuc-3’, from Eurogentec (Seraing, Belgique). Phosphorothioate bonds are indicated by subscript ‘s’. The miR-449 antagomiR targets Homo sapiens mIR-449a (full match) and miR-449b with one mismatch. The RRAS protector is a complementary reverse oligonucleotide targeting the conserved mIR-449 binding site of the human RRAS 3’-UTR. The antagomiR or protector negative control was the Clear-mIR-5’-cuaauauuucauaucuanuuu-3’, from Eurogentec. AntagomiR or antisense protector (100 μM) were pre-incubated with FCS for 30 min at room temperature. mIR-449a/antagomiR/FCS or protector/FCS mixture in 5’-auggcagagtacagcucaacgau-3’ and RRAS protector oligonucleotide (PO-RRAS: 5’-gugcaucagcaacagcuc-3’, from Eurogentec (Seraing, Belgique). Phosphorothioate bonds are indicated by subscript ‘s’. The miR-449 antagomiR targets Homo sapiens mIR-449a (full match) and miR-449b with one mismatch. The RRAS protector is a complementary reverse oligonucleotide targeting the conserved mIR-449 binding site of the human RRAS 3’-UTR. The antagomiR or protector negative control was the Clear-mIR-5’-cuaauauuucauaucuanuuu-3’, from Eurogentec. AntagomiR or antisense protector (100 μM) were pre-incubated with FCS for 30 min at room temperature. mIR-449a/antagomiR/FCS or protector/FCS mixture in 5’-auggcagagtacagcucaacgau-3’ and RRAS protector oligonucleotide (PO-RRAS: 5’-gugcaucagcaacagcuc-3’, from Eurogentec (Seraing, Belgique). Phosphorothioate bonds are indicated by subscript ‘s’. The miR-449 antagomiR targets Homo sapiens mIR-449a (full match) and miR-449b with one mismatch. The RRAS protector is a complementary reverse oligonucleotide targeting the conserved mIR-449 binding site of the human RRAS 3’-UTR. The antagomiR or protector negative control was the Clear-mIR-5’-cuaauauuucauaucuanuuu-3’, from Eurogentec (Seraing, Belgique). Phosphorothioate bonds are indicated by subscript ‘s’.
Ectopic expression of miRNAs or siRNAs. Cells were grown to 30% confluency in proliferation medium on plastic, glass coverslip or on Transwell filters. Cells were then transfected with synthetic negative control miRNA (miR-Neq, Ambion) or synthetic miR-449a/b miRNAs (Ambion) (10 nM final concentration). Total RNAs or proteins were extracted, or immunostaining was performed, from 24 to 72 h later. For siRNA experiments in differentiating HAEcs or AS49 cell line, cells were transfected with siRNA against the human RAS (si-RAS), ARHGD (si-ARHGD1) or ARHGA1 (si-ARHGA1) transcripts, or with a negative control siRNA (si-Neg) (Stealth RNA, siRNAs, Life Technologies) (20 nM final concentration) using Lipofectamine RNAi Max Reagent (Invitrogen) in OPTIMEM (Invitrogen) according to the manufacturer's instructions. On the next day, an additional transfection was performed using the same procedure, before HAE differentiation was induced in ALL on the third day. Finally, HAEcs were harvested for western blot analyses or processed for immunofluorescence experiments after 3 days for proliferating cells on glass coverslip or 7, 14 and 21 days for primary cultures in ALL.

Plasmid constructs and luciferase measurements. Sequence from the wild-type or mutants 3′-UTR of RAS, ARHGD1, ARHGA1, NDRG1 and DAAM1 were synthesized (gBlocks Gene Fragments, Integrated DNA Technologies) and cloned into psiCheck2 vector (Promega). For mutated 3′-UTRs, three bases of each seed region were changed by complementary bases. PsiCheck2 constructions were co-transfected with synthetic miRNA mimics (Ambion, Applied Biosystems) with or without antagonimiRs or antisense protectors into HEK293T cells, and luciferase activity was measured using the dual reporter luciferase assay kit (Promega), according to the manufacturer’s protocol.

ISH on Xenopus embryos. Whole-mount ISH analysis was done as follows: embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM MgSO4, 1 mM EGTA, 3.7% v/v formaldehyde) overnight at 4 °C, dehydrated in 100% ethanol or methanol overnight at 20 °C, then rehydrated in PBT (PBS + 0.5% Tween20), bleached for 5–10 min in H2O2, and hybridized overnight with the chosen probe. After hybridization, the embryos were washed at increasing stringency in SSC/10% CHAPS at 37 °C, then incubated overnight at 4 °C. After the blocking reagent, 15% goat serum or FCS. On the final day, embryos were extensively washed in MABX, then stained with Roche antibody in MABX, 2% Roche Blocking Reagent, 15% goat serum or FCS. On the next day, an additional transfection was performed using the same procedure, before HAE differentiation was induced in ALL on the third day. Finally, HAEcs were harvested for western blot analyses or processed for immunofluorescence experiments after 3 days for proliferating cells on glass coverslip or 7, 14 and 21 days for primary cultures in ALL.

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