Article

Bicc1 links the regulation of cAMP signaling in polycystic kidneys to microRNA-induced gene silencing

Nathalie Piazzon1, Charlotte Maisonneuve1, Isabelle Guilleret1, Samuel Rotman2, and Daniel B. Constam1,*

1 Ecole Polytechnique Fédérale de Lausanne (EPFL) SV ISREC, Station 19, CH-1015 Lausanne, Switzerland
2 Institute of Pathology, CHUV, Rue de Bugnon 25, CH-1011 Lausanne, Switzerland
* Correspondence to: Daniel B. Constam, E-mail: daniel.constam@epfl.ch

Genetic defects in autosomal-dominant polycystic kidney disease (ADPKD) promote cystic growth of renal tubules, at least in part by stimulating the accumulation of cAMP. How renal cAMP levels are regulated is incompletely understood. We show that cAMP and the expression of its synthetic enzyme adenylate cyclase-6 (AC6) are up-regulated in cystic kidneys of Bicc1−/− knockout mice. Bicc1, a protein comprising three KH homology (KH) domains and a sterile alpha motif (SAM), is expressed in proximal tubules. The KH domains independently bind AC6 mRNA and recruit the miR-125a from Dicer, whereas the SAM domain enables silencing by Argonaute and TNRC6A/GW182. Bicc1 similarly induces silencing of the protein kinase inhibitor PKια by miR-27a. Thus, Bicc1 is needed on these target mRNAs for silencing by specific miRNAs. The repression of AC6 by Bicc1 might explain why cysts in ADPKD patients preferentially arise from distal tubules.

Keywords: PKD, proximal tubules, bicaudal-C, cyclic AMP, PKA, PKια, miRNA

Introduction

MicroRNAs (miRNAs) curb the expression of a large fraction of the transcriptome. In general, primary transcripts (pri-miRNAs) are processed by the RNase III-like enzymes Drosha and Dicer into ~70-nucleotide stem-loop precursors (pre-miRNAs) and then into 21–23 bp duplexes that are loaded onto Argonaute (AGO) family proteins by the double-stranded RNA binding protein TRBP (Carthew and Sontheimer, 2009). AGOs unwind and retain the guide strands that recognize target mRNAs by Watson–Crick base pairing, and they recruit GW182/TNRC6A or its paralog TNRC6B or TNRC6C to form miRNA-induced silencing complexes (miRISCs). TNRC6 potentiates silencing by sequestering poly(A) binding protein (PABP) from a translation initiation complex with elf4G (Tritscher et al., 2010), and by recruiting CCR4-NOT and Pan2–Pan3 deadenylase complexes (Chekulaeva and Filipowicz, 2009; Huntertung and Izaurralde, 2011). Deadenylation triggers mRNA decay (Behm-Ansment et al., 2006; Fabian et al., 2009, 2011; Braun et al., 2011; Chekulaeva et al., 2011), and P-body formation (Andre et al., 2005; Stoeccklin et al., 2006; Eulalio et al., 2007; Zheng et al., 2008). RISC components are guided to target mRNAs by seed sequences that are complementary to nucleotides 2–8 of the mature miRNAs. However, genome-wide analysis of miRNA:mRNA pairs showed that only a fraction of predicted seed sequences are functional (Chi et al., 2009). In cultured cells and in the zebrafish germ line, access of miRNAs to specific targets can be modulated by RNA-binding proteins (RBPs) (Chekulaeva and Filipowicz, 2009; Agami, 2010; Muddashetty et al., 2011). It is possible, therefore, that RBPs also regulate miRNA silencing in somatic tissues.

Autosomal-dominant polycystic kidney disease (ADPKD) is characterized by fluid-filled cysts in kidneys and the pancreas that are usually linked to mutations in PKD1/poly cystin-1 or PKD2/poly cystin-2 (Torres and Harris, 2009). Poly cystin-2 is a Ca2+-permeable cation channel that is activated in ciliated renal epithelial cells by poly cystin-1 in response to fluid flow (Nauli et al., 2003; Praetorius and Spring, 2003; Xu et al., 2007a). Ca2+ reduces the synthesis of cAMP by adenylate cyclase AC6 to attenuate the activity of protein kinase A (PKA) and of the related kinase PRKX (Chabardes et al., 1999; Masyuk et al., 2006). PRKX stimulates the formation of glomeruli and ureteric branching during kidney development (Li et al., 2005b). Depending on subcellular localization by regulatory subunits, PKA and PRKX may phosphorylate multiple substrates, including ion channels, components of the Wnt, TGFβ, and Hh signaling pathways, and regulators of actin polymerization (Howe, 2004; Beene and Scott, 2007). The relative contributions of PKA versus PRKX and of candidate substrates to disease progression remain unknown.
Polycystic diseases also develop in mice and humans carrying mutations in the bicaudal-C (Bic-C) homolog 1 (Cogswell et al., 2003; Kraus et al., 2011). Bic-C enables head formation in Drosophila (Mohler and Wieschaus, 1986) by confining Oskar expression in oocytes to the posterior (Mahone et al., 1995; Saffman et al., 1998), and it modulates poly(A)-tail length of a subset of maternal mRNAs by binding the CCR4-NOT/CAF1 deadenylase (Chicoine et al., 2007). Bic-C contains five repeats of an RNA-binding K homology (KH) domain and a sterile alpha motif (SAM) (Mahone et al., 2003). The SAM concentrates BicC in cytoplasmic foci outside P-bodies and inhibits Dishevelled (Dvl) signaling in the canonical Wnt/β-catenin pathway (Maisonneuve et al., 2009). The substitution of glutamate E932 by glycine in the SAM domain of human BICC1 associates with pediatric unilateral renal dysplasias (Kraus et al., 2011). BicC is also mutated in the jcpk and bpk mouse models of PKD (Cogswell et al., 2003). In the jcpk allele, mutation of the splice acceptor of exon 2 introduces a truncation after the first KH domain (Cogswell et al., 2003). Homozygous mutants die postnatally showing bilateral cystic kidneys and dilated pancreatic and bile ducts, whereas 25% of aged heterozygotes develop glomerulocystic disease. In the bpk allele, a GC insertion in exon 22 elongates the C-terminus of the SAM domain. Targeted deletion of Bicc1 revealed an additional role in establishing left–right asymmetry (Maisonneuve et al., 2009). The positioning of visceral organs requires activation of motile cilia on posterior notochord cells (Tabin and Vogan, 2003). To generate flow, cilia are aligned by planar cell polarity (PCP) cues (Hashimoto and Hamada, 2010). In Bicc1 mutants, motile cilia fail to align, possibly due to excess Dvl/β-catenin signaling at the expense of PCP (Maisonneuve et al., 2009). However, the RNA-binding KH domains of Bicc1 are not required to inhibit Dvl signaling. Direct target RNAs and their regulation by Bicc1 therefore remain to be investigated.

Here, we identify AC6 and PKIα mRNAs as the first direct Bicc1 targets, and we show that Bicc1 is necessary for their loading unto AGO2 by miR-125a and miR-27a, respectively. Our findings link the deregulation of cAMP signaling in Bicc1 mutant polycystic kidneys to impaired miRNA-mediated gene silencing.

Results

**Bicc1 protein in proximal renal tubules maintains filamentous actin**

To investigate the role of Bicc1 in renal morphogenesis, we examined its distribution in wild-type (WT) and Bicc1−/− (Bicc1KO) kidneys. Neonatal mutant kidneys developed multiple cysts arising from Bowman’s capsule and proximal tubules akin to those of jcpk homozygotes and of mice carrying another targeted allele (Supplementary Figure S1) (Flaherty et al., 1995; Tran et al., 2010). In situ hybridization also confirmed the presence of Bicc1 mRNA in both cortical and medullary renal tubules (Figure 1A) (Tran et al., 2010). By contrast, Bicc1

---

**Figure 1** Bicc1 protein is expressed in proximal renal tubules and necessary to maintain filamentous actin. (A) **RNA in situ** hybridization of Bicc1 mRNA in neonatal WT and Bicc1KO kidneys. Bicc1 mutant mRNA (Maisonneuve et al., 2009) was undetectable since the antisense probe was complementary to the deleted region. (B) Frozen sections of postnatal kidneys of WT and Bicc1KO mice labeled with anti-Bicc1 antibody. Cortical regions are shown at higher magnification at the bottom. (C) Co-labeling with Lotus Tetragonolobus lectin (LTL) at P4 shows that the Bicc1 staining localizes to proximal tubules. LTL staining of mutant kidney sections at P0 (right panels) confirms that cysts develop in proximal tubules. Cryosections of WT and Bicc1KO kidneys at E15.5 (D) and P0 (E) labeled by TRITC-phalloidin (red) and DAPI (blue). Arrows in enlarged areas highlight F-actin. Scale bar, 100 μm.
immunostaining specifically labeled cortical epithelial structures (Figure 1B) reacting with the proximal tubule marker Lotus Tetragonolobus lectin (Figure 1C). In mutant kidneys, Lotus Tetragonolobus lectin stained cyst-lining cells, and the Bicc1 signal was lost (Figure 1C). The presence of brush borders confirmed that cysts of Bicc1 mutants originate from proximal tubules (Supplementary Figure S1A–J).

Cystic growth can be caused by defects in PCP (Saburi et al., 2008). To test whether PCP is generally impaired in Bicc1 mutants, we first analyzed cochlear hair cells in inner ears. Staining of stereociliary actin filaments and of acetylated tubulin in the kinocilium revealed normal planar polarization of hair cells in both WT and Bicc1KO neonates (Supplementary Figure S1K and L). By contrast, a striking loss of filamentous actin was apparent in cyst-lining epithelial cells of Bicc1KO kidneys already by the end of gestation, but not at earlier stages (Figure 1D and E). These results suggest that even though Bicc1 is not a core PCP gene, it is required in the kidney to maintain filamentous actin.

Polycystic kidneys of Bicc1 mutants accumulate cAMP and AC6 protein

In human ADPKD cells, altered actin dynamics, increased fluid secretion, and cell proliferation are linked to elevations in cAMP (Torres and Harris, 2006). In Bicc1KO neonatal kidneys, cAMP levels progressively increased from 1.7- to 9.5-fold compared with control littermates (Figure 2A). We therefore studied the regulation of ACs. Neither the level of AC6 mRNA nor the pattern of its polyadenylation was significantly altered in Bicc1KO compared with WT kidneys at any of the stages examined (Figure 2B and C). However, western blot analysis of kidney extracts at P0 revealed a 3.2-fold up-regulation of AC6 protein (Figure 2D). These data suggest that Bicc1 curbs AC6 expression independently of deadenylation.

Cyclic AMP activates the PKA and PRKX holoenzymes by binding regulatory subunits. To verify that PKA and PRKX are present, we independently of deadenylation.

Bicc1 binds AC6 mRNA and stimulates its translational silencing by miR-125a

Bicc1 may silence AC6 translation by recruiting specific miRNAs. To test this, we first asked whether Bicc1 can bind endogenous AC6 mRNA in HEK293T cells. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis revealed that HA-tagged Bicc1 coimmunoprecipitated AC6 mRNA as well as its own mRNA, whereas binding to β-actin control mRNA was below detection (Figure 3A). AC6 and Bicc1 mRNAs also co-precipitated with mutant HA-Bicc1ΔSAM lacking the SAM, but not with HA-Bicc1ΔKH lacking the KH domains. To test whether Bicc1 mediates silencing, the AC6 3′UTR was fused to luciferase cDNA and co-transfected with Bicc1 in HEK293T cells. Luciferase reporters containing a 1.5 kb fragment of the AC6 3′UTR or its proximal 448 bp were dose-dependently inhibited by WT Bicc1, but not by Bicc1ΔKH or Bicc1ΔSAM mutants (Figure 3B and Supplementary Figure S3A–C). In the proximal AC6 3′UTR, TargetScan and RNAfold analysis predict conserved seed sequences for miR-133, -365, -204/211, and -125/351 embedded in high-probability secondary structures (Figure 3C and Supplementary Figure S3D). A truncation lacking the miR-125/351 site, or mutation of only this seed sequence largely abolished silencing of the reporter by Bicc1 (Figure 3D). Bicc1-mediated silencing was similarly inhibited by an anti-miR neutralizing miR-125a (ASO125a), but not by control ASOs that target the passenger strand miR-125a*, or miR-96 (Figure 3E). We therefore tested whether Bicc1 also binds miR-125a. RT–PCR analysis revealed miR-125a in immunoprecipitates of HA-Bicc1 and HA-Bicc1ΔSAM, but not of HA-Bicc1ΔKH (Figure 3F). By contrast, no interaction was detected with the ubiquitous miR-16. These results suggest that Bicc1 represses the AC6 3′UTR by recruiting miR-125a.

To distinguish whether Bicc1 binds target mRNAs directly, or through cognate miRNAs, we monitored its association with mutant 3′UTR luciferase reporters. HA-Bicc1 co-immunoprecipitated with both Luc-AC6 3′UTRprox mRNA and the KO125 mutant (Figure 3G, lanes 5 and 7), whereas the truncation mutant 8125 failed to bind (Figure 3G, lane 6). These results suggest that 3′UTR sequences distal to the miR-125 site, rather than miR-125a, mediate binding of AC6 mRNA to Bicc1.

![Figure 2](https://academic.oup.com/jmcb/article-abstract/4/6/398/848951/figT1)

**Figure 2** Bicc1−/− mutant kidneys accumulate excess amounts of cAMP and AC6 protein. (A) Levels of cAMP in Bicc1−/− compared with WT littermate at stages P4 (n = 2 per genotype, P = 0.026), P7 (n = 7 per genotype, P = 10−4), and P11 (n = 2 per genotype, P = 0.09). (B) Relative expression levels of AC6 mRNA in WT and Bicc1KO kidneys as determined by qRT–PCR analysis at different stages (n = 3 per genotype and per stage). 100% corresponds to the expression in WT kidneys at P0. No significant alterations were detected at E17.5 (P = 0.27), P0 (P = 0.15), P2 (P = 0.17), or P4 (P = 0.58). (C) Reverse ligation PCR analysis of AC6 mRNA poly(A) tail length in embryonic and postnatal WT and Bicc1KO kidneys. (D) Western blot analysis of AC6 in kidney extracts of WT, heterozygous (HET), and Bicc1KO mice.
**Bicc1 inhibits expression of the PKA/PRKX inhibitor PKIα through miR-27a**

Free C subunits of PKA and PRKX can be inhibited by endogenous PKA inhibitors (PKIs). In chick embryos, PKIα is transiently down-regulated on the left side of Hensen’s node to enable asymmetric Nodal signaling during left–right axis formation (Rodriguez-Esteban et al., 2001). Therefore, we asked whether Bicc1 might also target PKIα. Quantitative RT–PCR revealed similar PKIα mRNA levels in Bicc1KO and WT newborn kidneys (Figure 4A). By contrast, western blot analysis of PKIα protein revealed a 4-fold up-regulation in mutants (Figure 4B, n = 4/4).

Conversely, overexpression of Bicc1 in HEK293T cells decreased the levels of endogenous PKIα protein, but not of its mRNA (Figure 4C). These results suggest that Bicc1 post-transcriptionally inhibits the expression of PKIα.

To investigate whether Bicc1 inhibits PKIα translation, we generated luciferase reporters containing the PKIα 3’UTR. Whereas the distal PKIα 3’UTR was unaffected by Bicc1, a proximal fragment conferred robust silencing by exogenous Bicc1, but not by Bicc1ΔKH or ΔSAM (Figure 4D, left panel). In this region, TargetScan predicts conserved seed sequences for miR-34, -27, and -23, but not for miR-125. To test the role of specific miRNAs, the 3’UTR luciferase reporter was co-transfected with anti-miR oligonucleotides or mutated in the corresponding seed sequences. Anti-miR-27a (ASO27a) as well as mutagenesis of the miR-27 seed (KO27) specifically abolished Bicc1-induced silencing, but had no effect in the absence of Bicc1 (Figure 4D, right panel). ASO27a also abrogated the repression of endogenous PKIα by Bicc1, whereas a control anti-miR (ASO96) had no effect (Figure 4E). Thus, Bicc1 and miR-27a mutually depend on each other to silence PKIα. PKIα mRNA and miR-27a also co-precipitated with full-length HA-Bicc1 and HA-Bicc1ΔSAM, but not with HA-Bicc1ΔKH (Figure 4F and G). Mutation of the miR-27a seed sequence did not inhibit binding of Luc-PKIα 3’UTR to HA-Bicc1 (Figure 4H). This confirms that Bicc1 binds but cannot silence target mRNAs independently of the recruitment of cognate miRNAs.

**Bicc1 KH domains bind specific miRNA precursors and associated Dicer**

With the first validated target RNAs at our disposal, we addressed why Bicc1 is required for their silencing. First, we tested whether Bicc1 acts before or after miRNA precursor processing. Immunoprecipitated HA-Bicc1 contained pre-miR-125a and -27a precursors, but not pre-miR-16 (Figure 5A). HA-Bicc1 and Dicer also co-precipitated in an RNase-resistant complex (Figure 5B and C). Binding of miRNA precursors and Dicer required the KH domains, but not the SAM. To validate these interactions, cell extracts containing endogenous Dicer were incubated with GST fusions of Bicc1 KH or SAM domains (Figure 5D). The KH domains, but not GST-SAM or empty beads pulled down Dicer (Figure 5E). This interaction was abolished if extracts were pre-treated with RNase prior to the addition of GST-KH-5, whereas RNase had no effect on preassembled complexes. We conclude that Bicc1 binds Dicer through RNA, which thus is sheltered from RNase. The acquired RNase resistance suggests that the bridging RNA is short, as befits specific miRNA precursors.
We next asked whether Bicc1 is required for miRNA precursor processing. Expression profiling of E17.5 kidneys after the onset of cyst formation showed that mIR-125a and -27a are abundantly expressed; however, the levels of mature miRNAs and the ratios between guide and passenger strands were not altered in Bicc1 mutants compared with WT (Table 1). We conclude that Bicc1 is required downstream of Dicer.

The SAM domain transfers Bicc1 target 3′UTR sequences to AGO2

Processed miRNA duplexes are unwound by AGO, which retains the guide strand to induce miRISC complexes with target miRNAs and TNRC6. To verify that silencing by Bicc1 requires miRISC, we depleted TNRC6A, AGO1, or AGO2 using specific siRNAs (Figure 6A). Bicc1-mediated silencing of AC6 and PKIα 3′UTR luciferase reporters thus was inhibited (Figure 6B). We therefore asked whether Bicc1 delivers target 3′UTR sequences to AGO2. In HEK293T cells, both endogenous AC6 mRNA and a full-length 3′UTR reporter co-immunoprecipitated with AGO2 even in the absence of Bicc1 (Figure 6C, lane 1). This is not surprising since the full-length 3′UTR harbors multiple conserved miRNA sites (Supplementary Figure S5) and can be targeted in HEK293T cells independently of Bicc1 by miR-96 and -182 (Xu et al., 2007b). By contrast, our reporter harboring the truncated proximal AC6 3′UTR co-precipitated with AGO2 only in the presence of Bicc1 (Figure 6C, compare lanes 2 and 6). Moreover, mutation of the miR-125 seed (lanes 7 and 8), or co-transfection of ASO125a (Figure 6D) prevented Bicc1 from loading the proximal AC6 3′UTR onto AGO2. By contrast, the control ASO133 had no effect. Interestingly, AGO2 loading of the proximal AC6 3′UTR also failed if Bicc1 lacked the SAM domain (Figure 6C, lanes 6 and 10). These results show that Bicc1 can transfer the proximal AC6 3′UTR to AGO2 in a process that requires a cognate miRNA and the SAM domain.
These results place Bicc as a novel determinant of miRNA target selection required for functional miRISC complexes. These observations identify Bicc as a vertebrate model. Bicc is evicted from target mRNAs prior to AGO loading, and it binds a fraction of TNRC6A that is not associated with target mRNAs and AGO in miRISC complexes. In summary, these results suggest that Bicc is required downstream of Dicer to recruit at least two novel target mRNAs, AC6, and PKIα into silencing complexes with specific miRNAs and AGO.

**Discussion**

Here, we have identified the first RNAs associating with Bicc in a vertebrate model. Bicc bound proximal AC6 and PKIα 3’UTR sequences and specific miRNA precursors such as pre-miR-125a and -27a, but not pre-miR-16 through its KH domains, whereas the SAM domain enabled their subsequent incorporation into functional miRISC complexes. These observations identify Bicc as a novel determinant of miRNA target selection required for regulating cAMP signaling. The levels of PKIα and AC6 proteins as well as cAMP increase in Bicc KO kidneys demonstrate that Bicc regulates these targets also in vivo.

**Role of miRNA in Bicc-mediated mRNA silencing**

In general, miRNAs guide AGO and associated TNRC6 proteins to target mRNAs to induce translational silencing and decay (Figure 6I). Only few RBPs have been found to regulate this process. In HeLa and NIH-3T3 cells, Drosophila and Dicer complexes contain the KH-type splicing regulatory protein, which is essential for the maturation of let7a miRNA (Trabucchi et al., 2009). Similarly in the brain, AGO1 and Dicer bind the fragile mental retardation protein (FMRP) (Caudy et al., 2002; Jin et al., 2004), and the loss of the Drosophila FMRP homolog impairs the processing of miR-124 precursor and the function of the miRNA bantam (Capurro et al., 2008; Yang et al., 2009). In cultured cortical neurons, FMRP also increases the levels of mature miR-20a, -125a, and Dicer bind the fragile mental retardation protein (FMRP) (Caudy et al., 2002; Jin et al., 2004), and the loss of the Drosophila FMRP homolog impairs the processing of miR-124 precursor and the function of the miRNA bantam (Capurro et al., 2008; Yang et al., 2009). In cultured cortical neurons, FMRP also increases the levels of mature miR-20a, -125a, and -27a, but not pre-miR-16 through its KH domains, whereas the SAM domain enabled their subsequent incorporation into functional miRISC complexes. These observations identify Bicc as a novel determinant of miRNA target selection required for regulating cAMP signaling. The levels of PKIα and AC6 proteins as well as cAMP increase in Bicc KO kidneys demonstrate that Bicc regulates these targets also in vivo.

**Table 1 Relative expression levels of selected mature miRNAs in Bicc WT, heterozygous and knockout kidneys at E17.5.**

<table>
<thead>
<tr>
<th>Rank</th>
<th>miRNA</th>
<th>wt</th>
<th>het</th>
<th>ko</th>
<th>SDhet</th>
<th>SDwt</th>
<th>SDko</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mmu-miR-720</td>
<td>15.37</td>
<td>15.27</td>
<td>15.27</td>
<td>0.00</td>
<td>0.18</td>
<td>0.00</td>
</tr>
<tr>
<td>15</td>
<td>mmu-miR-16</td>
<td>12.73</td>
<td>12.81</td>
<td>12.82</td>
<td>0.05</td>
<td>0.11</td>
<td>0.04</td>
</tr>
<tr>
<td>56</td>
<td>mmu-miR-27a</td>
<td>11.14</td>
<td>10.95</td>
<td>10.83</td>
<td>0.07</td>
<td>0.42</td>
<td>0.17</td>
</tr>
<tr>
<td>58</td>
<td>mmu-miR-125a</td>
<td>10.97</td>
<td>10.75</td>
<td>10.69</td>
<td>0.10</td>
<td>0.27</td>
<td>0.13</td>
</tr>
<tr>
<td>105</td>
<td>mmu-miR-17*</td>
<td>8.69</td>
<td>8.99</td>
<td>8.93</td>
<td>0.14</td>
<td>0.76</td>
<td>0.21</td>
</tr>
<tr>
<td>127</td>
<td>mmu-miR-17</td>
<td>8.07</td>
<td>8.65</td>
<td>8.42</td>
<td>0.07</td>
<td>0.97</td>
<td>0.30</td>
</tr>
<tr>
<td>234</td>
<td>mmu-miR-125a*</td>
<td>6.26</td>
<td>6.20</td>
<td>6.21</td>
<td>0.14</td>
<td>0.35</td>
<td>0.10</td>
</tr>
<tr>
<td>281</td>
<td>NegativeControl</td>
<td>5.01</td>
<td>4.74</td>
<td>5.03</td>
<td>0.46</td>
<td>0.48</td>
<td>0.52</td>
</tr>
<tr>
<td>537</td>
<td>mmu-miR-27a*</td>
<td>0.04</td>
<td>0.15</td>
<td>0.01</td>
<td>0.20</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>613</td>
<td>mmu-miR-16*</td>
<td>0.04</td>
<td>0.15</td>
<td>0.01</td>
<td>0.20</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>613</td>
<td>miRNABrightCorner30</td>
<td>7.58</td>
<td>7.59</td>
<td>7.53</td>
<td>0.22</td>
<td>0.49</td>
<td>0.31</td>
</tr>
<tr>
<td>537</td>
<td>DarkCorner</td>
<td>2.50</td>
<td>1.87</td>
<td>2.73</td>
<td>0.88</td>
<td>0.51</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*Ranking based on relative expression levels; Values represent the mean of the number of samples indicated in parentheses; *Passenger strands.

To ascertain that RISC formation was not an overexpression artifact, we analyzed miC3D cells that express endogenous Bicc (Fu et al., 2010). Anti-Ago2 antibodies specifically co-precipitated endogenous PKIα, AC6, and Bicc mRNA in cells transfected with control siRNA, but not in cells depleted of Bicc2 (Figure 6E). Transfection of miC3D cells with anti-miR-27a (ASO27a) or -125a (ASO125a) specifically abrogated Ago2 binding of PKIα and AC6 mRNAs, respectively (Figure 6F). These results place Bicc1 upstream of AGO.

To test whether Bicc1 activity depends on stable association with miRISC, we monitored binding of HA-tagged Bicc1 to AGO and TNRC6A. Co-immunoprecipitation analysis revealed no binding to AGO1 or AGO2, and only small amounts of TNRC6A co-precipitated with HA-Bicc1 independently of the KH or SAM domains even after RNase treatment (Figure 6G and H). We conclude that Bicc1 is evicted from target mRNAs prior to AGO2 loading, and that it binds a fraction of TNRC6A that is not associated with target mRNAs and AGO in miRISC complexes. In summary, these results suggest that Bicc1 is required downstream of Dicer to recruit at least two novel target mRNAs, AC6, and PKIα into silencing complexes with specific miRNAs and AGO.
miRNA duplexes to target mRNAs. The SAM domain enables subsequent transfer of Bicc silenced mRNAs. In the presence of Bicc mature miRNA duplexes directly to AGO, which recruits TNRC without binding detectable amounts of AGO |

Chekulaeva et al., 2011

| RAW TEXT END |

interaction is indirect. In line with this conclusion, pretreatment of cell extracts with RNase prevented pull down of Dicer by a GST fusion of the KH domains. However, microarray profiling of Bicc kidneys revealed no deficiency in miRNA maturation or stability. We therefore propose that Bicc is required downstream of Dicer to guide miRISC complexes to specific target mRNAs (Figure 6). Bicc co-precipitated immature miRNA precursors as well as mutant 3′UTRs lacking cognate seed sequences, indicating that these RNAs are recruited independently of each other. Subsequent transfer of target mRNA to AGO2 in addition required the Bicc SAM domain. Luciferase reporter assays confirmed that silencing by Bicc1 required the SAM, as well as TNRC6A, AGO1, and AGO2. The novel function of the SAM in RISC loading may be relevant in human patients, since a point mutation in this domain is associated with renal dysplasia (Kraus et al., 2011).

Our finding that depletion of either AGO1 or AGO2 blocked Bicc1 activity indicates that Bicc1 requires high levels of redundant AGO activities that share overlapping sets of targets (Hafner et al., 2010). Alternatively, depletion of AGO1 may derepress an unknown Bicc1 antagonist. Bicc1 facilitated AGO2 loading without binding detectable amounts of AGO1 or AGO2, suggesting that it is evicted during AGO2 loading. We propose that Bicc1 KH domains poise target mRNAs for silencing by inhibiting secondary structures or other factors that mask specific miRNA seed sequences, whereas regulatory input from the SAM domain enables subsequent loading unto AGO, in the simplest possible model by evicting Bicc1 (Figure 6). In keeping with this scenario, miR-125 and -27 sites in the 3′UTRs of Ac6 and PKlix are predicted to engage in stem loop structures (Supplementary Figure S3D, and unpublished data), and the probability of silencing inversely correlates with the stability of
such local structures (Kertesz et al., 2007; Hafner et al., 2010). Furthermore, our finding that Bicc1 binds the AC6 3′UTR within <105 nucleotides from the miR-125 seed is consistent with a role in regulating access to AGO.

We also tested whether Bicc1 can bind TNRC6A. Bicc1 and TNRC6A co-precipitated independently of KH and SAM domains. The absence of co-purified AGO proteins suggests that Bicc1 and AGO likely bind distinct pools of TNRC6. Binding of TNRC6A to Bicc1 may be mediated by CCR4-NOT, since Drosophila Bicc-C directly interacts with the CCR4-NOT3/5 subunit and promotes or inhibits deadenylation of oocyte mRNAs at different developmental stages without inducing mRNA decay (Chicoine et al., 2007). In our assays, the loss or gain of Bicc1 activity did not noticeably alter the levels or poly(A) tail length of target mRNAs. Bicc1 thus can uncouple the silencing of associated mRNAs from degradation, possibly by modulating access to decay enzymes.

**Novel target RNAs link Bicc1 to the regulation of PKA activity**

Previous studies established that cAMP agonists stimulate cyst expansion and transepithelial fluid transport in an *in vitro* model of renal cysts (Mangoon-Karim et al., 1989). Conversely, a pharmacological PKA/PRKX inhibitor blocks cAMP-induced proliferation of cystic ADPKD cells (Yamaguchi et al., 2000, 2003). Also, a vasoressin V2 receptor antagonist inhibiting cAMP synthesis diminishes cysts in rodent models of PKD (Gatton et al., 2003; Torres et al., 2004) and entered phase III clinical trials (Harris and Torres, 2009). However, insights into the mechanisms leading to elevated levels of cAMP are limited. In the *pck* rat model of PKD, several phosphodiesterases are post-transcriptionally inhibited (Wang et al., 2010), whereas in Bicc1 mutants, increased cAMP correlates with a de-repression of AC6. Interestingly, Bicc1 protein was only detected in proximal tubules, but not in collecting ducts. This may explain why collecting ducts in ADPKD patients are more prone to cystic growth than other segments.

**Autoinhibition and effect of Bicc1 on paracrine factors**

In *Drosophila*, Bicc-C mediates robust autoinhibition (Chicoine et al., 2007). Although mouse Bicc1 also interacts with its own mRNA, a *Bicc1lacZ* reporter allele is post-transcriptionally inhibited in the medulla even in the absence of functional Bicc1 (Tran et al., 2010), indicating that autoinhibition is not responsible. The localization of Bicc1 protein in proximal tubules concurs with the origin of cysts in that location in Bicc1 mutants. Thus, while we do not exclude that Bicc1 is expressed in other segments at low levels or at later stages, perinatal *Bicc1−/−* kidneys are expected to up-regulate direct targets such as AC6 and PKCx in proximal tubules. We were unable to test this prediction due to the lack of antibodies suitable for immunostaining (unpublished data). However, our model does not preclude a cell non-autonomous role for paracrine downstream effectors. Indeed, up-regulation of the secreted cytokine TGFcx is partly responsible for cystic growth in *Bicc1bpk/bpk* mice (Dell et al., 2001), and the loss of Bicc1 also affects Bowman’s capsules and collecting ducts (Tran et al., 2010; Supplementary Figure S1).

**Role of Bicc1 in the maintenance of filamentous actin**

A striking feature of the cystic kidneys of Bicc1 mutants is that they fail to maintain filamentous actin. F-actin staining also appears to be reduced in cyst-lining renal epithelial cells in *inv/inv* kidneys (Phillips et al., 2004) and in the *bpck* mouse model of Meckel syndrome type 3 (Cook et al., 2009). We are unaware of other reports on the distribution of F-actin in cystic kidneys. However, collapse of actin filaments in cultured cells inhibits the conductance of polycystin-2 (Li et al., 2005a). The activity of polycystin-2 channels is also inhibited in isolated cilia of renal epithelial cells treated with recombinant PKI (Raychowdury et al., 2009). Thus, both a decrease in F-actin and up-regulation of PKCx could contribute to cyst formation.

Actin dynamics are regulated by multiple inputs including PKA (Howe, 2004), PCP, and canonical Wnt signals that are integrated by small GTPases of the Rho family (Schlessinger et al., 2009). A role for Bicc1 in PCP seemed possible, since it inhibits Dishevelled activity in the cytoplasm (Maisonneuve et al., 2009). However, our finding that *Bicc1−/−* mice show no defect in the alignment of stereociliary bundles in cochlear hair cells does not support a general role in PCP. A major future challenge will be to assess whether the actin cytoskeleton of Bicc1KO renal epithelial cells is disrupted by a tissue-specific defect in PCP, by ectopic PKA/PRKX signaling, or by an unrelated mechanism. Distinguishing between these possibilities is beyond the scope of this study, since the pathways targeted by Bicc1 are interconnected and likely influence actin dynamics by multiple mechanisms in parallel. This complexity may also account for the fact that in other contexts Bicc1 inhibited the accumulation of F-actin, rather than stimulating it. Specifically, depletion of Bicc1 in cultured mIMCD3 cells induces actin stress fibers (Fu et al., 2010), and *bicc* mutant *Drosophila* oocytes accumulate ectopic F-actin cages around early recycling endosomes that diminish the release of the TGFcx homolog Grk (Kugler et al., 2009). *Bicc1* mutant kidneys, on the other hand, hyperactivate TGFcx (Dell et al., 2001) correlating with a decrease in F-actin.

**Role of miRNA in PKD**

Our results establish that Bicc1 inhibits the translation of AC6 and PKCx by joining these mRNAs to cognate miRNAs. A role for miRNAs in suppressing cystic growth in the kidney is plausible, since deletion of a floxed *Dicer* allele by Hoxb7::Cre in ureteric bud derivatives leads to dilatation of the renal pelvis and cortical cyst formation (Pastorelli et al., 2009). In a cholangiocyte cell line of the *pck* rat model and in polycystic liver disease patients, cyst formation has been linked to down-regulation of miR-15a (Lee et al., 2008). A general reduction in miRNA expression levels has been reported in the *cy/+* rat model of PKD (Pandey et al., 2008). However, information on the role of miRNAs in PKD and other kidney diseases is limited (Saal and Harvey, 2009). Our findings suggest that important insights in this area might be obtained by studying the interactions of specific miRNAs with Bicc1 and its target mRNAs and by testing whether they are de-regulated in PKD patients and preclinical models that show elevated levels of cAMP.

**Materials and methods**

**Bicc1 mutant mice**

Mice heterozygous for a targeted allele of *Bicc1* (Maisonneuve et al., 2009) were maintained on a C57BL/6 genetic background in individually ventilated cages at the EPFL animal facility.
Conserved miRNA seed sequences were predicted by TargetScan (http://www.targetscan.org/vert_50/). To generate miRNA reporters, 3’UTR fragments of mouse Ac6 or PKlx were inserted into a linker sequence between Phatinus pyralis luciferase cDNA (GenBank X84848) and 3’MS2 coat protein binding sites in the expression vector pcS2+ (sequences available upon request). In brief, a 1780 bp SalI–BamHI fragment comprising the 3’ end of the open reading frame and 1495 bp of the 3’UTR of an Ac6 EST clone (IMAGp998A0714048Q) was inserted in Xhol–BglII sites. The resulting construct was digested with PstI and religated to derive Ac6 3’UTRprox lacking the distal part of the Ac6 3’UTR. The mir-125 seed sequence CTCAAGGG was deleted or mutated to CTCAAGG by overlap extension PCR. CS-Luc-PKl3’UTRprox comprised an EcoRv–Scal 925 bp fragment of the EST clone IRAVpb968B06101D. A PshAl–Xbdl 1831 bp fragment of the same EST comprising the distal 3’UTR was subcloned to generate CS-LucPKI 3’UTRdist. The mir-27 seed sequence ACTGGTAA was mutated to ACTAgTAA. Sequences of siRNA duplexes (Microsynth) and transfection protocols for siRNAs and anti-miRTM miRNA inhibitors (Applied Biosystem, Ambion) are detailed in Supplementary Materials and methods.

Western blot and immunoprecipitation

Transfected cells or frozen kidneys were lysed in Laemmli buffer by ultrasonication on ice and centrifuged to remove debris. Custom-made affinity-purified anti-Bicc1 antibodies were raised in two rabbits co-injected with two immunogenic peptides (Eurogentec). Other antibodies used are listed in Supplementary Materials and methods. Densitometric analysis of immunoblots and normalization to γ-tubulin expression employed ImageJ software. To monitor protein–RNA interactions, immunoprecipitation was conducted on kidney cell lines (miMDC3 and 293T). HEK293T cells were transfected in 10 cm dishes using jetPEI™ (Polyplus Transfection). Two days after transfection, cells were washed with PBS, resuspended in extraction buffer (10 mM Tris–HCl, pH 7.4, 2.5 mM MgCl2, and 100 mM NaCl) supplemented with 0.02% NP-40 and protease inhibitors (Roche). After brief sonication and centrifugation at 9500 g for 15 min, supernatants were passed through a 0.2 μm filter and incubated for 2 h at 4°C with anti-HA beads (Sigma) or with anti-AGO2 or pre-immune IgG immobilized on G-Sepharose beads. Immuno-precipitates extracted with buffer-saturated phenol were treated with DNase I (10 U; Ambion) for 15 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. Equal amounts of samples were reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences) or Quantimir™ RT kit (System Biosciences). Primer sequences and protocols for PCR are detailed in Supplementary Materials and methods.

Polyadenylation assay

Polyadenylation was monitored by RNA ligation-coupled RT–PCR as described (Rassa et al., 2000). Briefly, 4 μg of total RNA was ligated to 0.4 μg of P1 anchor primer in a 10 μl reaction using T4 RNA ligase (New England Biolabs). The entire product was reverse transcribed by Superscript III (Invitrogen) in a 50 μl reaction using 0.4 μg of P1’ reverse primer. One microliter of the resulting cDNA was amplified by Platinum Taq (Invitrogen) in a 50 μl PCR reaction by annealing P1’ and a transcript-specific primer during 46 cycles at 56°C in the presence of 1.5 mM Mg2+. Transcript-specific primers annealed 50 nucleotides upstream of the poly(A) addition site. Where indicated, RNA samples were treated with oligo(dT) and RNaseH prior to ligation with P1’ oligonucleotide to verify that the PCR product is a specific readout of poly(A) tail elongation.

cAMP measurements

Kidneys of P4, P7, and P11 mice of Bicc1−−/− and WT littermates were weighed, grinded to fine powder in liquid nitrogen, and homogenized individually in 10 volumes of cold 0.1 M HCl in a glass-Teflon tissue grinder. After centrifugation at 600 g for 10 min at room temperature, equal volumes of supernatant were individually processed without acetylation using the Format A Cyclic AMP ‘PLUS’ Enzyme Immunoassay Kit (Biomol International).

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

Acknowledgements

We thank Drs Robert Kotin (NIH Bethesda, USA) and Gunter Meister (Univ. Regensburg, Germany) for anti-Prkx antisemur and anti-AGO2 antibody and to Dr Andrew Dudley (Northwestern Univ., Evanston, USA) for advice on cochlear stainings. We also thank Drs Mihaela Zavolan (Biozentrum, Basel, CH, Switzerland), Gregory Goodall (Univ. Adelaide, Australia), and Bernhard Rossier (Univ. Lausanne, CH, Switzerland) for comments on the manuscript.

Funding

This work was supported by the American PKD Foundation (151a2) and by the Swiss National Science Foundation (Sinergia) (CRSII3_130662).

Conflict of interest: none declared.

References


Chabardes, D., Imbert-Teoul, M., and Elalouf, J.M. (1999). Functional properties of Ca2+-inhibitable type 5 and type 6 adenylyl cyclases and role of Ca2+...
increase in the inhibition of intracellular cAMP content. Cell. Signal. 11, 651–663.


