ATMIN is a transcriptional regulator of both lung morphogenesis and ciliogenesis

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ABSTRACT
Initially identified in DNA damage repair, ATM-interactor (ATMIN) further functions as a transcriptional regulator of lung morphogenesis. Here we analyse three mouse mutants, Atmingpgg6/pgg6, AtminH210Q/H210Q and Dynll1GT/GT, revealing how ATMIN and its transcriptional target dynein light chain LC8-type 1 (DYNLL1) are required for normal lung morphogenesis and ciliogenesis. Expression screening of ciliogenic genes confirmed Dynll1 to be controlled by ATMIN and further revealed moderately altered expression of known intraflagellar transport (IFT) protein-encoding loci in Atmin mutant embryos. Significantly, Dynll1GT/GT embryonic cilia exhibited shortening and bulging, highly similar to the characterised retrograde IFT phenotype of Dynll1. Depletion of ATMIN or DYNLL1 in cultured cells recapitulated the in vivo ciliogenesis phenotypes and expression of DYNLL1 or the related DYNLL2 rescued the effects of loss of ATMIN, demonstrating that ATMIN primarily promotes ciliogenesis by regulating Dynll1 expression. Furthermore, DYNLL1 as well as DYNLL2 localised to cilia in puncta, consistent with IFT particles, and physically interacted with WDR34, a mammalian homologue of the Chlamydomonas cytoplasmic dynein 2 intermediate chain that also localised to the cilium. This study extends the established Atmin-Dynll1 relationship into a developmental and a ciliary context, uncovering a novel series of interactions between DYNLL1, WDR34 and ATMIN. This identifies potential novel components of cytoplasmic dynein 2 and furthermore provides fresh insights into the molecular pathogenesis of human skeletal ciliopathies.

KEY WORDS: Asciz, Atmin, Ciliogenesis, Ciliopathy, Dynll1, Mouse

INTRODUCTION
Cilia are membrane bounded microtubule-based extensions of the centrosome that demonstrate varied roles in mammalian development and adult physiology (Satir and Christensen, 2007; Quinlan et al., 2008; Baker and Beales, 2009; Norris and Grimes, 2012). Motile cilia are requisite to normal respiratory and reproductive tract function, left-right (L-R) patterning and ependymal cell function (Quinlan et al., 2008). Immobile primary cilia, present on most cells, are involved in wide-ranging functions that include cell-cell signalling, force perception, sensation and cell polarity (Satir and Christensen, 2007; Baker and Beales, 2009; Oh and Katsanis, 2012).

Proteomic, genomic and genetic analyses have revealed hundreds of proteins to be present in cilia (Gherman et al., 2006). These are transported into and out of cilia by a process termed intraflagellar transport (IFT) (Kozminski et al., 1993). IFT particles moving toward the ciliary tip (anterograde) and those moving back towards the cell (retrograde) are driven by kinesin 2 and cytoplasmic dynein 2 motors, respectively (Rosenbaum and Witman, 2002; Pedersen and Rosenbaum, 2008). Whereas the components of kinesin 2 are well known, the constitution of cytoplasmic dynein 2 remains uncertain (Pfister et al., 2006). In comparison to six proteins identified as comprising cytoplasmic dynein 1, only two proteins (DYNC2H1 and DYNC2L1) have been definitively identified for mammalian cytoplasmic dynein 2 (Pfister et al., 2006). In Chlamydomonas reinhardtii, the cytoplasmic dynein 2 complex is known to contain at least five different subunits (Rompolas et al., 2007; Patel-King et al., 2013), suggesting that other components remain to be identified for mammalian cytoplasmic dynein 2. In addition to motors and cargo, IFT particles also comprise IFT proteins that organise into two different complexes, IFT-A and IFT-B, with roles in anterograde and retrograde transport as well as ciliary trafficking of membrane proteins (Goetz and Anderson, 2010; Pedersen and Christensen, 2012). Mutations in IFT or motor protein that prevent anterograde transport lead to loss of overt cilia, whereas those affecting retrograde IFT are associated with shortened and often malformed cilia (Pedersen and Rosenbaum, 2008; Goetz and Anderson, 2010). The crucial role of both motile and immotile (primary) cilia in mammalian development and disease have become increasingly evident (Satir and Christensen, 2007; Quinlan et al., 2008; Baker and Beales, 2009; Norris and Grimes, 2012). Complete loss of cilia results in early embryonic lethality (Norris and Grimes, 2012).
whereas postnatal cilia loss leads to obesity and polycystic kidney disease (Davenport et al., 2007). A variety of cilia-based defects underlie both embryonic lethal and adult viable human syndromes, including Jeune syndrome (JS), Meckel-Grübler syndrome (MKS), Joubert syndrome (JBTS), short-rib polydactyly syndrome (SRPS), Bardet-Biedl syndrome (BBS) and Alström syndrome (ALS). Collectively these are known as the ciliopathies (Badano et al., 2006). Initially defined through the overlapping signs and symptoms of a small collection of disorders (Badano et al., 2006), this group has expanded, and continues to expand, through systematic analysis of overlapping syndromes (Baker and Beales, 2009) and model organism genetics (Norris and Grimes, 2012). Intriguingly, individual genes can underlie multiple ciliopathies; variations in allele strength determine the precise syndromic outcome (Hildebrandt et al., 2011). However, evidence has also been advanced for multilocus-driven ciliopathies (Burghes et al., 2001; Katsanis et al., 2001), and it is easy to see how an allele at a second locus could influence the severity of a ciliopathy. Indeed, studies in mouse have revealed how heterozygous mutations in IFT loci can influence the severity of DynclI mutants, acting to suppress aspects of the phenotype (Ocbina et al., 2011). While many of the genes involved in ciliogenesis remain to be identified, it is evident that ciliary defects underlie a significant number of developmental human disorders (Baker and Beales, 2009). Although a core set of ciliopathy signs and symptoms has been defined, these are not present in every ciliopathy. Indeed, rarer defects are evident in only a proportion of ciliopathies, while severe developmental defects incompatible with life will a priori occur only in lethal ciliopathies. Deficiencies in pulmonary development and patterning have been reported for a subset of ciliopathies: pulmonary hypoplasia is described for some SRPS (MIM: 263520) and patterning have been reported for a subset of ciliopathies: only in lethal ciliopathies. Deficiencies in pulmonary development defined, these are not present in every ciliopathy. Indeed, rarer developmental human disorders (Baker and Beales, 2009). Suppress aspects of the phenotype (Ocbina et al., 2011). While regulates ciliogenesis (Pazour et al., 1998), we hypothesised that ATMIN can result from cilia dysfunction; we were therefore intrigued by the reported lung phenotype of a mouse mutant in ATMIN-activator (ATMIN, also known as ASCIZ) (Jurado et al., 2010).

RESULTS

ATMIN function is required for normal development

We identified two mouse point mutants (Atmin<sup>gpg6</sup> and Atmin<sup>H210D</sup>) in the ATMIN ZFs. Both resulted in an embryonic lethal phenotype highly similar to that of the null mutant (Atmin<sup>−/−</sup>) and reminiscent of a ciliopathy. Indeed, analysis of embryonic nodes, limb buds and neural tubes revealed shortened cilia in Atmin mutant embryos. ATMIN zinc fingers are required for transcription factor activity (Jurado et al., 2012b); analysis of embryonic gene expression revealed modest, but consistent, reductions in FoxJ1, Ifi88, Ifi172 and Ifi140 expression in the mutants. As expected, a highly significant drop in Dynll1 expression was also detected, although its close parologue Dynll2 remained unaffected. Analysis of Dynll1 mutant embryos revealed a gross embryonic phenotype highly similar to Atmin. Shortened nodal cilia were evident and demonstrated a bulging morphology highly reminiscent of cytoplastic dynein 2 mutants. Knockdown of Atmin or Dynll1 in cultured cells resulted in almost identical phenotypes; fewer ciliated cells and reduced ciliary length, a phenotype rescued by overexpression of Dynll1 or Dynll2. Both Dynll1 and Dynll2 exhibited ciliary localised puncta, consistent with IFT particles, as might be predicted for cytoplastic dynein 2. Analysis of hedgehog signalling revealed this to be downregulated tissue specifically in the developing lungs. We further demonstrated the putative dynein intermediate chain, WDR34, to localise to cilia and to directly interact with Dynll1. Together, these data support the argument that ATMIN regulates DYNNLI1, which in turn controls retrograde IFT and ciliogenesis. Thus, these data provide evidence of an ATMIN-DYNNLI1 pathway controlling ciliogenesis.

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In order to confirm our previous findings (Ermakov et al., 2009), a congenic C3.C-gpg6 strain was generated, segregating away additional mutations and providing a defined, homogeneous genetic background. Analysis of homozygous C3.C-gpg6 embryos revealed a similar gross phenotype to that we previously reported: combinations of oedema, exencephaly, pulmonary hypoplasia and pulmonary situs anomalies (Table 1; Fig. 1E-H). However, in contrast to the variability of lung size (full sized, small or absent) seen in the outbred stock, only small lungs were evident (Fig. 1H), all of which...
demonstrated abnormal situs (Table 1). The low level micrognathia we previously reported was no longer obvious, but craniofacial defects remained evident, most obviously in the form of a thickening of the philtrum (Table 1). Both cardiac and gut situs were wild type, but abnormal cardiac outflow tract development was evident in almost all embryos (supplementary material Table S1).

Atmin has a well-characterised role in DNA damage pathways (Kanu and Behrens, 2008). In order to assess whether Atmingpg6/gpg6 embryonic phenotypes might relate to a deficiency in the DNA damage response, we analysed the intracellular localisation of the DNA damage marker 53BP1 in wild-type (supplementary material Fig. S3A) and mutant embryos (supplementary material Fig. S3B). Relocalisation of 53BP1 into discrete nuclear foci is an accepted DNA damage marker (Fig. S3A) and mutant embryos (supplementary material Fig. S3B). However, we were unable to detect any 53BP1 foci in wild-type (supplementary material Fig. S4).

Strikingly, the number of very short (<1.5 μm) cilia present in Atmingpg6/gpg6 nodes significantly outnumbered that seen in wild type (Fig. 2L). Limb bud cilia from wild-type and mutant embryos were similarly analysed (Fig. 2D,E), and although there was a far smaller variability in these innately short cilia, a small but significant drop (P = 0.011; Fig. 2G) in cilia length was evident in Atmingpg6/gpg6. Similar analysis of neural tube (NT) cilia (Fig. 2H,I) again revealed a statistically significant reduction in cilia length (P = 0.027; Fig. 2K). The nature of the Atmingpg6 mutation is such that it could conceivably encode a dominant-negative protein. No heterozygous phenotype was, however, evident in embryos or adults (data not shown) and when node cilia in heterozygous Atmin+/gpg6 nodes were analysed, no obvious changes in cilia length were detected (supplementary material Fig. S4).

## Abnormal hedgehog signalling in ATMIN mutant lungs

The hedgehog (HH) signalling pathway is coordinated by primary cilia, and defective cilia are known to have an impact on HH signalling (Goetz and Anderson, 2010); previously described phenotypes include polydactyly and neural tube patterning. However, overt limb patterning was unaffected in Atmingpg6/gpg6 embryos (Fig. 1). Similarly, when dorsoventral patterning of Atmingpg6/gpg6 and control NTs was analysed, highly similar results were obtained (Fig. 3A). Cleavage of GLI3 protein is diagnostic of HH signalling. In order to assess HH signalling in the embryo, the ratio of cleaved to uncleaved GLI3 protein was examined. In wild-type and Atmingpg6/gpg6 embryos, both full-length and cleaved GLI3 repressor (Gli3R) were detected (Fig. 3B); small, but statistically significant variations between wild-type and mutant samples were evident, implying slightly increased HH signalling (Fig. 3C). Together, these data support the argument that although minor changes to HH signalling are present in Atmingpg6/gpg6 mutants, they are insufficient to have an impact on limb or NT patterning.

Defective HH signalling is known to affect lung patterning and can give rise to phenotypes similar to those that we describe for Atmingpg6/gpg6 (Motoyama et al., 1998). It seemed possible that defects in HH signalling were restricted to certain tissues, such as the lungs. We therefore collected embryonic lungs, but due to the small size of Atmingpg6/gpg6 lungs we were unable to readily assess GLI3 cleavage. Both Ptc1 and Gli1 are, however, regulated by HH and their expression provides a readout of HH signalling. When we assessed their expression by qPCR, we found Ptc1 expression in Atmingpg6/gpg6 mutants to be ∼80% of wild-type levels and Gli1 expression to be less than 50% of wild type (Fig. 3D). These results were consistent and statistically significant over 11
mutant lung samples, demonstrating reduced HH signalling in Atmingp6/gpg6 lungs.

ATMIN regulates expression of ciliogenic genes
The ATMIN protein localises to nuclei and functions as a transcriptional regulator (McNees et al., 2005; Kanu and Behrens, 2007), but it has not been reported in, and we do not detect it in, the cilium (data not shown). We therefore performed a directed expression screen to assess expression of known IFT-associated loci in 11.5 days post coitum (dpc) wild-type and Atmingp6/gpg6 embryos. When expression of the ciliogenic transcription factors, Rfx3 and FoxJ1, was analysed, no significant difference in Rfx3 expression was detected. However, a small, but statistically significant reduction in FoxJ1 expression was evident in the mutant (P<0.05; Fig. 4A). Expression of four IFT loci was similarly analysed; two IFT-A (Ift140, Ift112) and two IFT-B (Ift88, Ift172) genes (Fig. 4B). A small but clear (~0.3-fold) downregulation of Ift88, Ift172 and Ift140 was evident in Atmingp6/gpg6 mutants when compared with wild-type expression. By contrast, Ift122 expression remained equivalent between mutant and wild-type samples.

Expression of Kif3a and Kif3b, coding for subunits of the anterograde IFT motor heterotrimeric kinesin 2, showed no significant variation between wild-type and mutant samples (Fig. 4C). The known mammalian dynein 2 components Dync2h1 and Dync2li were similarly examined and no statistically significant expression differences were evident between mutant and wild-type samples (Fig. 4C). The two mammalian LC8 homologues, Dynll1 and Dynll2, show strong similarity at the amino acid level, differing by only six out of 89 amino acids. Although the proteins are indistinguishable by current antibodies, differences in mRNA sequence allow their expression to be differentiated. Evidence from cell lines shows that ATMIN can regulate expression of Dynll1 (Jurado et al., 2012b); we detected a 17-fold decrease in Dynll1 expression in Atmingp6/gpg6 mutant embryos compared with wild-type littermates (Fig. 4C), consistent with ATMIN similarly controlling Dynll1 expression in the embryo. By contrast, the levels of Dynll2 expression were equivalent between mutant and wild-type embryos (Fig. 4C).

The relationship between ATMIN and Dynll1 expression was confirmed when ATMIN was overexpressed in mouse inner medullary collecting duct (IMCD3) cells. Transient transfection...
of an Atmin-expressing construct resulted in a 12-fold increase in the level of Atmin mRNA over untransfected cells (supplementary material Fig. S5). Consistent with Atmin regulating Dynll1 expression, Dynll1 mRNA levels increased by 30-fold compared with controls (supplementary material Fig. S5). Thus, Atmin directly or indirectly regulates Dynll1, but not Dynll2, expression in embryos.

Dynll1, a potential retrograde IFT mutant recapitulating Atmin

Our analysis suggested a pathway whereby Atmin function affects expression of Dynll1, and to a lesser extent a number of IFT protein loci, which in turn have an impact on ciliogenesis. The strong downregulation of Dynll1 expression suggests that loss of DYNLL1 function should recapitulate significant elements of the Atmin<sup>gpg6/gpg6</sup> phenotype, both at the developmental and the cellular levels. In order to test this, we sourced a genetrap allele of Dynll1 [Dynll1<sup>Gt(EUCE0287d04)Hmgu</sup>, hereafter Dynll1<sup>Gt/Gt</sup>]. Analysis of mRNA levels in homozygous Dynll1<sup>Gt/Gt</sup> embryonic tissue demonstrated highly significant downregulation of Dynll1 expression, consistent with it being a functional null allele (supplementary material Fig. S6). Analysis of 13.5 dpc Dynll1<sup>Gt/Gt</sup> embryos revealed a complex phenotype similar to that of Atmin<sup>gpg6/gpg6</sup> mutants (Fig. 1); gross oedema, exencephaly and coloboma were evident (Fig. 1M-P, Table 1). Small, mispatterned lungs were detected in almost 70% of the embryos examined (Table 1), with the remainder demonstrating no lungs. Similar to Atmin<sup>gpg6/gpg6</sup> pulmonary isomerism was evident in a significant proportion of Dynll1<sup>Gt/Gt</sup> embryos (Table 1).

Analysis of cardiac outflow tract development revealed high incidence of interrupted aortic arch and common outflow tract development, similar to the results seen for Atmin<sup>gpg6/gpg6</sup> mutants (supplementary material Table S1). Strikingly, additional situs defects were evident in Dynll1<sup>Gt/Gt</sup> embryos, with high incidence of reversed heart and stomach situs (Table 1). Analysis of nodal cilia from Dynll1<sup>Gt/Gt</sup> revealed these to be shorter than wild-type controls (Fig. 2B,L), although the phenotype was less extreme than in Atmin<sup>gpg6/gpg6</sup> nodes (Fig. 2B,L). However, these cilia showed a bulging morphology, with a high incidence of bulges around the base of the cilia (Fig. 2C), a phenotype highly reminiscent of that reported for Dyncl2h1 mutant embryos (Huangfu and Anderson, 2005; Ocbina et al., 2011). Examination of limb bud and neural tube cilia revealed no significant changes in cilia length, but did reveal changes in cilia morphology with bulges and/or fattening of the cilia evident (Fig. 2F,J; supplementary material Fig. S7D-H). These defects are highly characteristic of defective retrograde IFT (Satir et al., 2010). To further assess this phenotype we derived mutant (Dynll1<sup>Gt/Gt</sup>) and wild-type embryonic fibroblasts. We analysed IFT protein localisation by

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**Fig. 2. ATMIN and DYNLL1 are required for normal ciliogenesis.** Tissue from stage-matched wild-type (A,D,H), Atmin<sup>gpg6/gpg6</sup> (B,E,I) and Dynll1<sup>Gt/Gt</sup> (C,F,J) embryos imaged by SEM. (A-C) Two- to four-somite stage embryonic nodes; insets show higher magnification of one cilium from the main panel. (D-F) Limb bud cilia at 11.5 dpc. (H-J) Neural tube cilia at 11.5 dpc. (G) Comparative analysis of limb bud cilia length (n=75, three embryos per class). (K) Comparative analysis of neural tube cilia length (n=450, three embryos per class). *P=0.027, **P=0.0106. Dynll1<sup>Gt/Gt</sup> cilia width was substantially increased in both limb bud (F) and neural tube (J) (see supplementary material Fig. S7 for statistical analysis). (L) Distribution of nodal cilia length is shifted towards shorter cilia in Atmin<sup>gpg6/gpg6</sup> and Dynll1<sup>Gt/Gt</sup> mutants (n=500, three embryos per class).
staining for the IFT protein IFT88. This revealed greatly increased staining in *Dynll1* 

**Fig. 3.** Perturbed HH signalling in *Atmin* mutant lungs. (A) Dorsoventral patterning of 10.5 dpc *Atmin* and wild-type neural tubes assessed by NKKX2.2, PAX6, FOXA2 and Olig2 immunostaining. No obvious differences are evident. (B) Western blot analysis of 9.5 dpc embryo extracts shows increased full-length 190 kDa GLI3 isoform (GLI3FL) and decreased 83 kDa cleaved repressor form (GLI3R) in *Atmin* embryos when compared with wild-type controls. A GLI3+− embryo extract confirms the specificity of the anti-GLI3 antibody. β-tubulin was used as a loading control. (C) Quantification of the results reveals a 30% increase GLI3FL and a 20% decrease in the levels of GLI3R in the mutant embryos. (D) Hedgehog signalling was assessed by qPCR expression analysis for *Gli1* and *Ptc1*, as well as for the known target gene *Dynll1*. There is statistically significant reduction in Gli1 expression *P*<0.01 and *Ptc1* expression *P*<0.05 (Anova).

**DYNNL2 rescues ciliogenesis defects in cells lacking Atmin**

In light of these results on the *Atmin* and *Dynll1* mutants, we then investigated the involvement of both proteins in mammalian ciliogenesis in vitro. IMCD3 cells produce clearly discernible cilia in culture (Fig. 6A); using short-interfering RNA (siRNA), we individually knocked down *Atmin* and *Dynll1*. Consistent with the analysis of *Atmin* embryos, *Atmin* knockdown disrupted ciliogenesis (Fig. 6B). A similar effect was observed upon *Dynll1* knockdown (Fig. 6C). The proportion of ciliated cells dropped from 78.8% in controls, to 55.7% and 56.8% when *Atmin* and *Dynll1*, respectively, were knocked down (Fig. 6D). More strikingly, the length of the remaining cilium was reduced following knockdown, with a control length of 3.6 μm being reduced to 1.2 μm and 1.8 μm following *Atmin* and *Dynll1* knockdown, respectively (Fig. 6E). When mRNA from the siRNA-mediated knockdown of *Atmin* was analysed, both *Atmin* and *Dynll1* mRNA were found to be highly downregulated (Fig. 6F). Importantly, overexpression of either MYC-DYNLL1 or MYC-DYNLL2 rescued the *Atmin* knockdown phenotype, leading to an almost threefold increase in the number of ciliated cells in the *Atmin* knockdown (Fig. 6G). A similar fold increase was observed in *Dynll1* knockdown cells when either MYC-DYNLL1 or MYC-DYNLL2 was expressed, demonstrating that DYNLL2 can rescue at least aspects of DYNLL1 loss. Together, these data provide strong evidence for *Atmin* regulating *Dynll1* expression, which in turn regulates ciliogenesis.

**DYNNL1 localises to cilia and interacts with the putative dynein intermediate chain WDR34**

Although the full constitution of the mammalian cytoplasmic dynein 2 remains uncertain (Pfister et al., 2006), the *Chlamydomonas* DYNNL1/2 homologue LC8 is known to comprise part of the retrograde IFT dynein motor (Pazour et al., 1998; Rompolas et al., 2007). We therefore postulated that DYNNL1 is likely to comprise a component of mammalian cytoplasmic dynein 2. If this were the case, DYNNL1 must be present within cilia. Due to their high similarity, no isoform-specific antibodies exist for DYNNL1 and DYNNL2. We therefore stained for the presence of LC8 (DYNNL1 and DYNNL2) within nodal cilia, finding it to be present (Fig. 7A). In *Chlamydomonas* LC8 is also known to be a component of axonemal dynein (Piperno and Luck, 1979; Pfister et al., 1982; King and Patel-King, 1995) and as such might be expected to be uniformly present along the length of the motile nodal cilia. A nonmotile fraction of nodal cilia, however, lacks axonemal dynein (McGrath et al., 2003). Our findings show all cilia within the node to be positive for LC8 staining (supplementary material Movie 1 and Fig. S9); moreover, the staining was punctate, suggesting localised concentrations, consistent with IFT particles.

To further confirm these data we analysed cilia in serum-starved IMCD3 cells. These immortal primary cilia lack axonemal dynein (Satin and Christensen, 2007), allowing the presence of LC8 protein within the IFT-dynein to be more easily assessed. We again detected the presence of LC8 in cilia, in addition to a strong signal at the base...
of cilia (Fig. 7B); puncta within the nucleus were also evident. The same result was found in NIH3T3 cells (supplementary material Fig. S10). In order to distinguish DYNLL1 and DYNLL2 we visualised MYC-DYNLL1 and MYC-DYNLL2 expressed in IMCD3 cells (Fig. 7C, D). The myc tag was detected in the nucleus and at the base of cilia, as well as within the ciliary axonemes for both DYNLL1 and DYNLL2, consistent with both proteins existing within cytoplasmic dynein 2. A highly similar axonemes for both DYNLL1 and DYNLL2, consistent with both proteins existing within cytoplasmic dynein 2. A highly similar

DISCUSSION

In this study we demonstrate a novel pathway that affects ciliogenesis, comprising ATMIN and its transcriptional target Dynll1. The strong similarity in cilia phenotype between Dynll1 and Dync2h1 mutants supports the argument that loss of Dynll1 protein has an impact on cytoplasmic dynein 2 function, an interpretation further supported by the interaction of Dynll1 and WDR34. The documented role of Dynll1 in mammalian cytoplasmic dynein-1 (Pfister et al., 2006) and in the Chlamydomonas retrograde dynein motor (Pazour et al., 1998) reinforce this model. This reveals a novel role for ATMIN, outside of the DNA damage pathway where it was first described (McNees et al., 2005), as a transcriptional regulator of both ciliogenesis and lung morphogenesis.

Two engineered null alleles of Atmin have been generated (Jurado et al., 2010; Kanu et al., 2010), and the description of their phenotypes strongly overlaps Atmin<sup>gpg6</sup> embryos. However, all these mutants are embryonic lethal and as such postnatal roles of ATMIN, in for example DNA damage and cancer (Loizou et al., 2011), have not been easily assessed. Certain differences are evident between the reported phenotypes of the Atmin<sup>−/−</sup> and Atmin<sup>gpg6</sup>, Atmin<sup>−/−</sup> embryos survived to a greater age than Atmin<sup>gpg6</sup>, although these differences may relate to differing genetic background. Here we report significant cardiac outflow tract defects, a deficiency likely underlying lethality; the cardiac outflow tract is not explicitly commented on in the Atmin<sup>−/−</sup> analyses (Jurado et al., 2010; Kanu et al., 2010). At the cellular level, studies of Atmin<sup>−/−</sup> embryos did not report defects in ciliogenesis; however, cilia were not explicitly examined. While we see strong agreement in phenotype, we cannot rule out differences between the different alleles. The point mutations that we describe should affect DNA binding, and hence transcription factor activity, but should not have an impact on regions of the protein involved in ATM or CHK2 interaction. It is easy to imagine that phenotypes associated with ATM and CHK2 function could differ significantly between Atmin<sup>−/−</sup> and Atmin<sup>gpg6</sup> mutants; investigation of these potential differences await future studies.

The skeletal ciliopathies, which include SRPS, Sensenbrenner syndrome and JS, manifest as defects that include shortened limbs, thoracic constriction, retinal degeneration, cystic kidneys and congenital heart disease (Huber and Cormier-Daire, 2012). Incidence of lung mispatterning is associated with some members of the group (MIM: 263520, 208500). Strikingly, mutations in loci affecting retrograde IFT underlie a significant proportion of these disorders; mutations in the cytoplasmic dynein 2 heavy chain, Dync2h1, and the IFT-A genes Ift122, Dync35/Ift121 and Ift43 have been reported (Huber and Cormier-Daire, 2012). Recently it has emerged that mutations in the putative cytoplasmic dynein 2 intermediate chain WDR34 underlie JS (Huber et al., 2013; Schmidts et al., 2013). Human disease mutations are often hypomorphic, making direct comparison to mouse loss-of-function
alleles challenging. For example, human DYNC2H1 point mutations lead to viable SRPS and JS individuals; in contrast, a mouse Dync2h1 allele results in embryonic lethality and a phenotype grossly similar to Atmingpg6, albeit with polydactyly and more severe L-R patterning defects. Similar mouse phenotypes result from mutations in the IFT-A loci Ift122 (Cortellino et al., 2009) and Ift121/Wdr35 (Mill et al., 2011); strikingly, the Wdr35yeti mutant demonstrates pulmonary hypoplasia. Other indications of the relationship between cilia and lung patterning have emerged from the study of mouse ciliopathy models (Ermakov et al., 2009; Weatherbee et al., 2009).

Pulmonary agenesis and hypoplasia are relatively rare phenotypes that have previously been reported as a result of defective HH, Wnt/beta-catenin and FGF signalling (Min et al., 1998; Motoyama et al., 1998; Li et al., 2002; Shu et al., 2002; Rajagopal et al., 2008; Goss et al., 2009). The HH pathway is known to require normal cilia to function (Goetz and Anderson, 2010), and we have demonstrated a clear impact on HH signalling in Atmingpg6 lungs; in light of these results and the obvious phenotypic overlap with the lungs of HH pathway mutants (Litingtung et al., 1998; Motoyama et al., 1998), it seems most likely that defective HH signalling underlies the pulmonary defects that we report. However, ciliopathies are by their nature pleiotropic, so it is pertinent to ask how the other pathways might be affected by cilia. A substantial literature exists on the relationship between cilia and canonical Wnt signalling (Wallingford and Mitchell, 2011); although controversy remains on this subject, it is clear that some ciliopathies do have an impact on the canonical Wnt pathway (Wallingford and Mitchell, 2011). FGF receptors have been reported to localise to cilia in the mouse node (Tanaka et al., 2005), raising a possible role for cilia in FGF signalling. FGF signalling is known to affect cilia length control (Neugebauer et al., 2009); however, the lack of other overt FGF phenotypes and the impact of Dynll1GT on ciliogenesis supports the argument against this being the cause of cilia phenotypes in Atmingpg6.

The striking similarity in phenotype between Atmin and Dynll1 mutants suggests that much of the Atmingpg6 phenotype manifests through regulation of Dynll1 expression. However, differences exist between the two mutants. In terms of our analysis, the most striking difference is in cilia morphology. There is strong downregulation of Dynll1 expression in Atmingpg6/gpg6 embryos, but not total loss of expression. The remaining low levels of Dynll1 may permit sufficient retrograde IFT to prevent the basal bulging of cilia. This alone might explain why Dynll1GT/GT mutants do not phenocopy Atmingpg6 mutants. A second possibility exists in that ATMIN regulates not only Dynll1 expression, but also expression of other loci; we have demonstrated downregulation of Ift88, Ift172, Ift140 and FoxJ1.

A recent study revealed that loss of one copy of either an IFT-A (Ift122) or IFT-B (Ift172) locus partially rescues the Dync2h1 phenotype, leading to loss of similar bulges from cilia (Ocbina et al., 2011). In the case of Atmingpg6 at least three IFT loci are downregulated, including Ift172. In light of the similarity of phenotype between Dynll1 and Dync2h1 we cannot rule out a genetic interaction between targets of ATMIN; downregulation of Dynll1 expression being partially rescued by downregulation of IFT loci.

One of the most striking results of this study is the presence of bulges at the base of Dynll1GT/GT cilia. Conventional understanding of IFT suggests that failure of retrograde transport should result in...
bulges at the tips of cilia, a phenotype reported for certain IFT-A mutants (Tran et al., 2008; Ocbina et al., 2011). It would be tempting to postulate that the basal bulges in Dynll1<sup>GT/GT</sup> embryos reflect functions of DYNLL1 outside of cytoplasmic dynein 2, were it not for the published data on Dync2h1 (Ocbina et al., 2011). Both Dynll1<sup>GT/GT</sup> and Dync2h1<sup>l/l</sup> embryos show bulges at the base of cilia, suggesting that this phenotype can represent defective cytoplasmic dynein 2 function. This could reflect partial loss of cytoplasmic dynein 2 function, caused by hypomorphic alleles, and/or partial functional redundancy; our data suggest that DYNLL2 may be able to function in place of DYNLL1. These data, however, suggest a role for cytoplasmic dynein 2 in the efficient exit of IFT particles from the base of the cilium. The apparent differences between IFT-A and cytoplasmic dynein 2 mutant cilia also make it unclear whether cytoplasmic dynein 2 is the only retrograde motor functioning in the cilium. Further genetic analysis of definitive null alleles of these and additional loci may cast light on this. Unfortunately, the published analysis of Dync2h1 mutants, the other characterised cytoplasmic dynein 2 component, provides insufficient resolution to fully assess ciliary morphology (Rana et al., 2004).

Our analysis reveals a dual role for ATMIN in both ciliogenesis and DNA damage pathways. Indeed, ciliopathy causing mutations in genes encoding DNA damage proteins, have previously been described (Chaki et al., 2012; Zhou et al., 2012). The existence of such links is not entirely unexpected, as cilia must be dismantled, releasing the centrioles before cells divide (Satir and Christensen, 2007); phosphorylation of the dynein light chain TCTEX1 is pivotal in both ciliary disassembly and cell cycle progression in ciliated cells (Li et al., 2011). Following DNA damage, cell cycle checkpoints function to prevent cell division and it seems possible that a similar function is being mediated through preventing (or simply slowing) cilia from being dismantled. The complete absence of both cilia and centrioles, however, does not affect normal DNA damage responses (Bazzi and Anderson, 2014). A contrasting hypothesis is that such interactions might sustain cilia in DNA damaged cells, maintaining the ability of such cells to receive cilia-dependent signals. Whether such interactions interlink ciliogenesis and the cell cycle remains to be determined.

In summary, we present data demonstrating a role for ATMIN transcriptional regulation of Dynll1 in ciliogenesis. Our data further provide evidence for DYNLL1 and WDR34 comprising elements of the mammalian cytoplasmic dynein 2, taking the number of identified components from two to four. The rescue of ATMIN ciliogenesis defects by DYNLL1 reflects the major role played by this transcriptional relationship. Evidently both loci have additional well-defined roles. The variations in phenotype between the Atmin and Dynll1 mutants demonstrate that transcriptional regulation of ciliogenesis is not necessarily a simple linear process.

Fig. 6. DYNLL1 expression rescues the in vitro ciliogenic defects caused by loss of ATMIN. (A) Control IMCD3 cells form normal cilia on serum starvation. (B) siRNA-mediated knockdown of Atmin results in reduced cilia number and reduced cilia length. Following siRNA-mediated knockdown of Dynll1 both the frequency and the length of cilia is strongly reduced (C). Quantitation of changes in percentage ciliated cells (D) and mean cilia length (E). (F) qRT-PCR analysis showing decreased Atmin and Dynll1 expression (relative to β-actin) upon Atmin knockdown (kd); expression is normalised to mock knockdown controls (crtl). (G) Overexpression of DYNLL1-myc and DYNLL2-myc rescue the Atmin and Dynll1 knockdown ciliary phenotype compared to scrambled siRNA and mock control experiments.
between rs13480001 and rs13480012 and candidate sequencing identified by et al., 2009). SNP-based haplotypic mapping defined a minimal region on the ENU-derived EA-Mice (G) Co-expression of Wdr34-GFP and myc-Dynll2: immunoprecipitation (IP) with myc co-precipitates a band detectable with anti-GFP antibodies. (F) Co-expression of Wdr34-GFP and myc-Dynll1: immunoprecipitation (IP) with GFP co-precipitates a band detectable with anti-myc antibodies and IP with myc co-precipitates a band detectable with anti-GFP antibodies. (E) GFP-WDR34 localises to the ciliary axoneme. (D) myc-DYNLL1 (C) and myc-DYNLL2 (D) localise to the base of the cilium, and to the ciliary axoneme. (B) AtminH210Q is isolated from the Harwell ENU archive (Quwailid et al., 2004). Both strains were maintained by backcrossing to C57Bl6HeH. All mice were housed in IVCs in the Mary Lyon Centre. Dynll1 and Dynll2 embryos, 12.5 dpc, were collected. For positive control, wild-type embryos were placed in 0.025% methyl methanesulfonate (Sigma, 129925) in DMEM medium (Gibco) for 3 h. Embryos were fixed in 4% PFA, ethanol dehydrated and paraffin embedded. 5 μm sections were taken, deparaffinised, rehydrated and subjected to heat-induced antigen retrieval at 110°C for 2 min in buffer containing 10 mM sodium citrate, 0.05% Tween-20, pH 6.0. The staining was carried out using EnVision G2 Doublestain System (Dako). The 53BP1 antibody (cat. A300-272A, Bethyl Laboratories) was used at a dilution of 1:5000 for 1 h at room temperature. For colour development, 3,3′-diaminobenzidine tetrahydrochloride (DAB+′) and Hematoxylin were used. The slides were scanned using Aperio ScanScope scanner and analysed using ImageScope software (both Aperio Technologies). Protein preparation, western blotting and immunoprecipitation Epitope-tagged constructs were transfected into HEK-293T cells using JetPei (PolyPlus Transfection) according to the manufacturer’s instructions. Immunoprecipitations (IPs) and western blots were performed as previously described (Field et al., 2011). In brief, HEK-293T cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche) and prepared protein was quantified using Bradford reagent (Sigma). Protein was resolved with 4-12% Bis-Tris gels (Life Technologies), transferred onto nitrocellulose membranes (iBLot; Life Technologies), then blocked with 5% milk in PBT (0.1% Tween-20 in PBS). Membranes were probed with anti-DLC8 (1:10,000; Abcam, Ab51603), anti-GFP (1:4000; Roche, ab3271) or anti-Gli3 [1:500 (Wen et al., 2010)] primary antibodies, fluorescently conjugated rabbit and mouse secondary antibodies (1:15,000; LI-COR Biosciences), then visualised with the Odyssey imaging system (LI-COR Biosciences). IPs were performed with 0.4 mg pre-cleared lysate per IP, using 1 μg of anti-GFP (Roche, 11,814,460,001) or anti-Myc (Sigma, C3956) antibodies bound to Protein G Sepharose beads (Sigma).

Cell culture, transfection and immunofluorescence IMCD3 cells were grown in DMEM/F12 (Gibco) media supplemented with 5% foetal bovine serum (Life Technologies), and pen-strep (Life Technologies) on 13 mm glass coverslips, thickness No. 0. NIH3T3 were similarly cultured but in DMEM/F12 media supplemented with 10% foetal bovine serum (Life Technologies). Constructs were transfected using JetPei (Polyplus Transfection) according to the manufacturer’s instructions. Between 6 and 18 h post transfection, growth medium was changed to 0% serum medium and cells were left ‘serum starved’ for 72 h, to encourage ciliation. Cells were pre-extracted for 30 s in 0.5% Triton X-100 in PHEM buffer (Schiele and van Blerkom, 1981), then fixed in 4% PFA in PHEM buffer at 37°C for 15 min. Embryos dissected in cold PBS were fixed in 4% PFA in PBS for 20 min at room temperature, then washed in 0.5% SDS in PBS three times, 10 min per wash. Staining for Myc and HA protein tags was with goat anti-Myc (1:200; Abcam, ab9132) and mouse anti-HA (1:100; Covance, MMS-191R). Cilia were labelled using anti-detyrosinated tubulin (Glu-tubulin, 1:200; Millipore, AB3201). Endogenous LC8 was detected using rabbit anti-LC8 (1:500; Abcam, ab51603) and cilia with an antibody against the mutation. AtminH210Q was isolated from the Harwell ENU archive (Quwailid et al., 2004). Both strains were maintained by backcrossing to C57Bl6HeH. All mice were housed in IVCs in the Mary Lyon Centre. Dynll1 and Dynll2 embryos, 12.5 dpc, were collected. For positive control, wild-type embryos were placed in 0.025% methyl methanesulfonate (Sigma, 129925) in DMEM medium (Gibco) for 3 h. Embryos were fixed in 4% PFA, ethanol dehydrated and paraffin embedded. 5 μm sections were taken, deparaffinised, rehydrated and subjected to heat-induced antigen retrieval at 110°C for 2 min in buffer containing 10 mM sodium citrate, 0.05% Tween-20, pH 6.0. The staining was carried out using EnVision G2 Doublestain System (Dako). The 53BP1 antibody (cat. A300-272A, Bethyl Laboratories) was used at a dilution of 1:5000 for 1 h at room temperature. For colour development, 3,3′-diaminobenzidine tetrahydrochloride (DAB+′) and Hematoxylin were used. The slides were scanned using Aperio ScanScope scanner and analysed using ImageScope software (both Aperio Technologies). Protein preparation, western blotting and immunoprecipitation Epitope-tagged constructs were transfected into HEK-293T cells using JetPei (PolyPlus Transfection) according to the manufacturer’s instructions. Immunoprecipitations (IPs) and western blots were performed as previously described (Field et al., 2011). In brief, HEK-293T cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche) and prepared protein was quantified using Bradford reagent (Sigma). Protein was resolved with 4-12% Bis-Tris gels (Life Technologies), transferred onto nitrocellulose membranes (iBLot; Life Technologies), then blocked with 5% milk in PBT (0.1% Tween-20 in PBS). Membranes were probed with anti-DLC8 (1:10,000; Abcam, Ab51603), anti-GFP (1:4000; Roche, 11,814,460,001), anti-Myc (1:1000; Sigma, C3956) anti-ATMIN (Millipore, ab3271) or anti-Gli3 [1:500 (Wen et al., 2010)] primary antibodies, fluorescently conjugated rabbit and mouse secondary antibodies (1:15,000; LI-COR Biosciences), then visualised with the Odyssey imaging system (LI-COR Biosciences). IPs were performed with 0.4 mg pre-cleared lysate per IP, using 1 μg of anti-GFP (Roche, 11,814,460,001) or anti-Myc (Sigma, C3956) antibodies bound to Protein G Sepharose beads (Sigma).
acetylated tubulin (1:500; Sigma, T7451), IFT88 (1:500; Abcam, ab42497). Visualisation utilised Dylight 488 mm and 650 mm secondary antibodies (1:250; Abcam, ab9635, ab96875, ab96894). Slides were visualised on an Axio Observer Z1 inverted microscope (Zeiss) fitted with an Apotome attachment, or a LSM700 confocal. For neural tube markers, Nkx2.2 (mouse; DSHB, 74.5A5), Olig2 (rabbit; Millipore, AB9610), Pax6 (rabbit; Millipore, AB2237), FoxA2 (goat; Santa Cruz, sc-6554X) were used.

siRNA-mediated knockdown

For RNAi knockdown siRNAs were transfected into ~60% confluent IMCD3 cells utilising Lipofectamine RNAiMax (Life Technologies), as previously described (Dawe et al., 2009). Post transfection, cells were serum starved for 96 h before staining and imaging. Knockdown was confirmed by qRT-PCR. Transfection efficiency was assessed using Block-iT Alexa Fluor Red Fluorescent Control (Invitrogen), confirming high level transfection. Pools of three siRNAs were used for Atmin; MSS214922, MSS214923 and MSS214924 (Invitrogen) and for Dynl1; S80610, S80611, S80612 (Invitrogen). Scrambled control was ON-TARGET plus Non-Targeting Pool, D-001810-10 (Thermo Scientific). Cilia number and length were measured manually from ten fields of view of >100 magnified images and statistical significance of differences between scrambled and Dynl1 and Atmin knockdowns was assessed using Student’s t-tests.

Quantitative PCR

One mg RNA was isolated from wild-type and Atmin+/pg6 glands. E11.5 littersates were provided by ABI. Quantitative real-time PCR was performed in triplicate on six different embryonic RNA pools of three siRNAs were used for Atmin; MSS214922, MSS214923 and MSS214924 (Invitrogen) and for Dynl1; S80610, S80611, S80612 (Invitrogen). Scrambled control was ON-TARGET plus Non-Targeting Pool, D-001810-10 (Thermo Scientific). Cilia number and length were measured manually from ten fields of view of >100 magnified images and statistical significance of differences between scrambled and Dynl1 and Atmin knockdowns was assessed using Student’s t-tests.

Electron microscopy

For morphological analysis of cilia, embryos, limb buds and neural tube portions were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated through an ethanol series, transferred into acetone, critical point dried (EMTECH 850), mounted, sputter coated with platinum (8 nm; QUORUM 150RS) and viewed in a JEOL 6010LV scanning electron microscope. Tissue was collected in parallel for genotyping. For nodes four wild-type and four mutant embryos were analysed, all visible cilia measured and plotted for frequency against length. For neural tubes four wild-type and four mutant samples were analysed; ~140 cilia were measured per sample. For limb buds three wild-type and three mutant samples were compared; between 50 and 150 cilia were measured per sample. Two-tailed t-test was used to test for statistical significance of differences.

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Competing interests

The authors declare no competing financial interests.

Author contributions

P.G., J.L.S., F.A., J.K., D.T.G., S.H.P., A.D., D.J.W., H.H., R.R., C.T.E. and N.P.-G. designed, performed and analysed experiments. A.E. identified the gpg6 mutant and performed initial genetic mapping. G.W. and C.A.J. performed and analysed knockdown experiments. J.S. advised on and helped with confocal and wide-field imaging. J.B. analysed neural tube patterning. S.K.M. and L.B.P. developed and tested WDR34 constructs. S.V.K. and G.L.D. performed DNA damage analysis. J.B., C.A.J. and L.B.P. provided further intellectual and conceptual input into the design of the study and writing of the manuscript. D.P.N. conceived the study and wrote the manuscript.

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Supplementary material

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