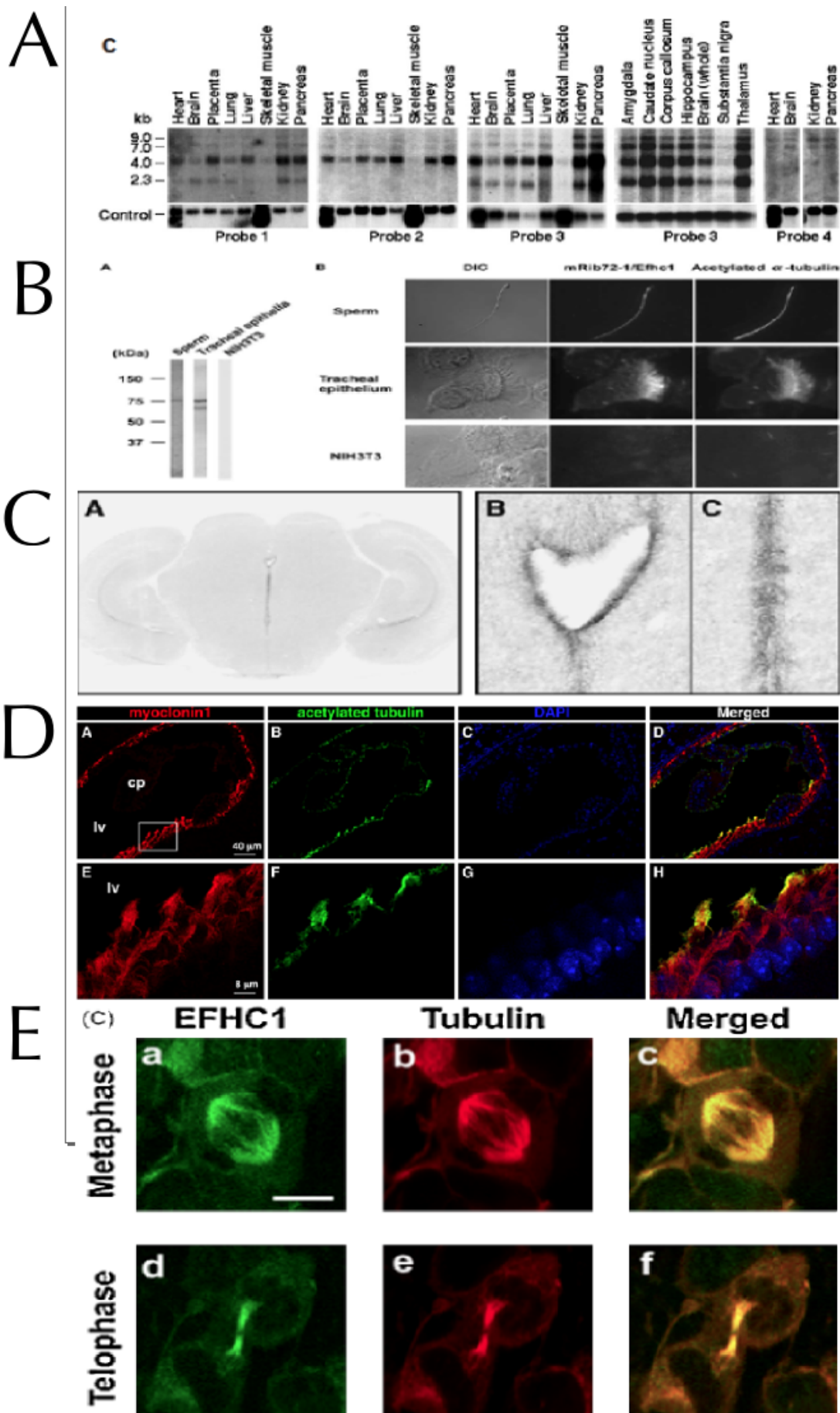


Knockdowns of axonemal proteins cause unexpected transcriptional defects: Supplemental Figures



Supplemental figure 1- *EFHC1* was detected in the axonemes of motile ciliated cells and the spindle fibers of most mitotic vertebrate cells.

A) Suzuki et Al. (2004) performed northern blots with 4 radioactive probes for mouse *EFHCs*. Probe 2 is specific for *EFHC2*, Probe 3 specific for *EFHC1*, and probe 4 is specific for an EST (sequences not shown). Suzuki et Al. also found through immunoprecipitation that Myc-tagged Myoclonin coprecipitates with R-type $\text{Ca}_v2.3$ calcium channel protein (50kd) even for cells transfected with mutant *Myc-EFHC1* (not shown). Immunohistochemistry showed that Myoclonin colocalized with MAP2 in hippocampal primary culture neurons, but this antibody was shown to be nonspecific when it localized to the ventricular ependyma in *-/-* mice (Suzuki 2008) (not shown).

B) Ikeda et Al. (2005) show that Myoclonin is targeted to interphase cells with motile cilia. Ba) Western blot for 100ug of total adult mouse protein shows that a polyclonal antibody reacts with a 75-kDa protein, which is the size of *EFHC1*/Myoclonin. Human NIH3T3 cells, a fibroblast cell line with primary cilia, were grown under conditions encouraging G_0 and therefore primary cilia; their extract does not react with the antibody, showing that Myoclonin is not present in primary-only ciliated cells. Another western blot in the paper shows that the antibody is somewhat less reactive with extracts from the brain and lungs, but not at all reactive with tissues lacking motile cilia (i.e. pancreas, liver, and the eye). Bb) DIC shows cell morphology: sperm are large cells with a long flagella; tracheal epithelium are round cells with dozens of flagella per cell; and NIH3T3 are round, amorphous cells with one small, antennalike primary cilium each. Fluorescence imaging of Cy3-linked antibody shows Myoclonin is present in the flagella of sperm and at the surface of tracheal epithelium, but not at all in NIH3T3 cells. For a positive control, signal for acetylated- α -tubulin is shown in all three cell types where it is a component of all cilia but not for cytoplasmic microtubules which have different post-translational modification.

C) Suzuki et Al. (2008) performed in-situ in coronal brain sections and showed *EFHC1* signal in ventricle walls and the central canal. Ca) Central coronal sections of E18 mouse cerebella showed signals in ventricular epithelium, which secrete cerebrospinal fluid which is circulated by motile cilia. Cb and Cc show detail from Ca. Some found these in-situ unconvincing despite the fact that the signal was not found in *EFHC-/-* brain samples. These in-situ signals were validated by RT-PCR in Leon et Al. (2009), also showing low levels of expression in non-ventricular cells. These low-levels signals were for glia without motile cilia.

1D) Suzuki et Al (2008) revisited the mouse brain with a new 6A3-mAb monoclonal anti-Myoclonin antibody in tissue sections of p14 mouse brains. In this paper, the antibody was

validated using *EFHC1*^{-/-} mice where signal was not found. Their new Myoclonin antibody binds the apical surface of ventricular cells of the choroid plexus (Da and De). Anti-acetylated- α -tubulin shows the coordinated axons of multiciliated cells (Db and Df). DAPI staining shows cell nuclei where Myoclonin1 is not found (Dc and Dg). Myoclonin1 antibody completely overlapped antibody for acetylated- α -tubulin (Dd and Dh) but not antibody for glial fibrillary acid protein (GFAP) or microtubule-associated protein 2 (MAP2) which is a MAP with well-understood binding to microtubules (not shown).

1E) Leon et Al. (2009) performed immunohistochemistry in neuroblast stem cells at different stages of the cell cycle. Myoclonin is found in concert with α -tubulin in metaphase cells (top row) and telophase (bottom row). This substantiates EFCH1/Myoclonin as an axonemal protein that is intimately involved with mitosis, perhaps with a role in neuronal migration (figure 11).

Supplementary video 1: *EFHC1*^{-/-} mice reach adulthood but experience spontaneous myoclonus.

http://hmg.oxfordjournals.org/content/suppl/2009/01/04/ddp006.DC1/ddp006_movie_1.mov

Supplementary video 2: Normal ciliary beating in ventricular ependyma of control mice.

http://hmg.oxfordjournals.org/content/suppl/2009/01/04/ddp006.DC1/ddp006_movie_2.mov

Supplementary video 3: impaired ciliary beating in ventricular ependyma of *EFHC1*^{-/-} mice.

http://hmg.oxfordjournals.org/content/suppl/2009/01/04/ddp006.DC1/ddp006_movie_3.mov

Supplemental Figure 2- My abbreviated in-situ protocol

0. Transfer the embryos from the original glass tube (where they are fixed and stored in EtOH) into a 1.5 ml glass tube then replaced the embryo stocks with EtOH.

1. Rehydrate the embryos through washing in MeOH/PBS

I did this by washing 5 mins each in MeOH/PBS mixtures:

100% MeOH

25% PBS: 75% MeOH

50% PBS: 50% MeOH

75% PBS: 25% MeOH

100 PBS: 0 MeOH (rehydrated)

2. Wash 3x in PBSw for 5 minutes. Fill vials.

3. Change to 10 ug/ml Proteinase K in PBSw (.5 ml per vial) and incubate for 15 minutes at room temperature (don't rock).

4. Short PBSw rinse.

5. Wash 2x w/ PBSw for 5 minutes each.

6. Refix in 4% PFA in PBSw. Let stand 5 min, then rock 15 min.

8 ml WATER

1 ml 10x MEM salts

1 ml 37% (stock) formaldehyde (so final= 3.7%).

7. Rinse in PBSw.

8, 9, 10, 11, 12. Wash 5x w/ PBSw, 5 min. each. Fill vials.

13. Add 1ml Prehyb heated to 65°C.

14. Prehybridize, rocking, at 60°C for 2-6 hours.

15. Heat approx. 200ng probe to 100°C for 4 min. Use the 1-4 ul probe only, do not dilute in hyb.

16. Remove the recycled prehyb, return it to the lab stocks, then add 1ml fresh prehyb (65°C) to the probe (95°C).

17. Add the fresh prehyb with probe to the embryos.

18. Incubate the probe/hyb at 60°C O/N. (Hybridization step).

Day 2

19. Save probe after hybridization (the probe improves in quality, often works best on the second or third use.)

20. Wash 2x in 2x SSC w/.1%CHAPS for 30 minutes

21. Rinse in .2x SSC

22. Wash 2x in .2xSSC w .1% CHAPS at 65°C for 30 minutes.

23. Short rinse in TBS w/ .1% Triton X-100 (=TBSX).

For each tube of embryos make .6 ml of antibody buffer:

For 1 ml:

800ul TBSX,

150 ul Horse serum

50ul Xenopus protein extracts

24. Incubate embryos in 500 ul antibody buffer for at least 3 hours at 4°C while shaking.

25. In parallel, preabsorb AP-conjugated anti-dig antibodies (for a final dilution of 1:3000) against an equivalent 100 ul antibody solution for 3-4 hours at RT.

26. Add 100ul of preabsorbed antibody solution to embryos (Final dilution of antibodies becomes 1/3,000).

27. Wash overnight at 4°C (cold room rocker).

Day 3- Wash out antibody

28. Short rinse w/ TBSX.

29. Wash 6x in TBSX for 1 hr at RT.

29b. Meanwhile, for each tube, rock 500 ul of BM Purple in the dark at RT for 1 hour.

30. Short rinse the embryos in Alkaline-Phosphatase buffer.

For 1ml:

100 ul Tris 9.5

20ul 5M NaCl

50 ul MgCl₂

830 ul H₂O

31. Rock embryos in 500ul AP-Buffer for 15 minutes at RT.

32. Replace AP Buffer with .5ml BM Purple

33. Develop embryos in 500 ul BM Purple in the dark.

Day 4- Stop the reaction. Often this would take place on the same day as the TBSX washes.

34. Stop the AP reaction by washing twice in PBS for 10 min ART.

35. Refix embryos in MEMFA for 2 hours.

36. Rinse with PBS then dehydrate through graded MeOH rinses:

100% PBS w/o MeOH

75% PBS: 25% MeOH

50% PBS: 50% MeOH

25% PBS: 75% MeOH

37. If you want to bleach, make a solution of 1 ml bleach per tube:

10%: 30% H₂O₂,

5%: Formamide,

85%: MeOH

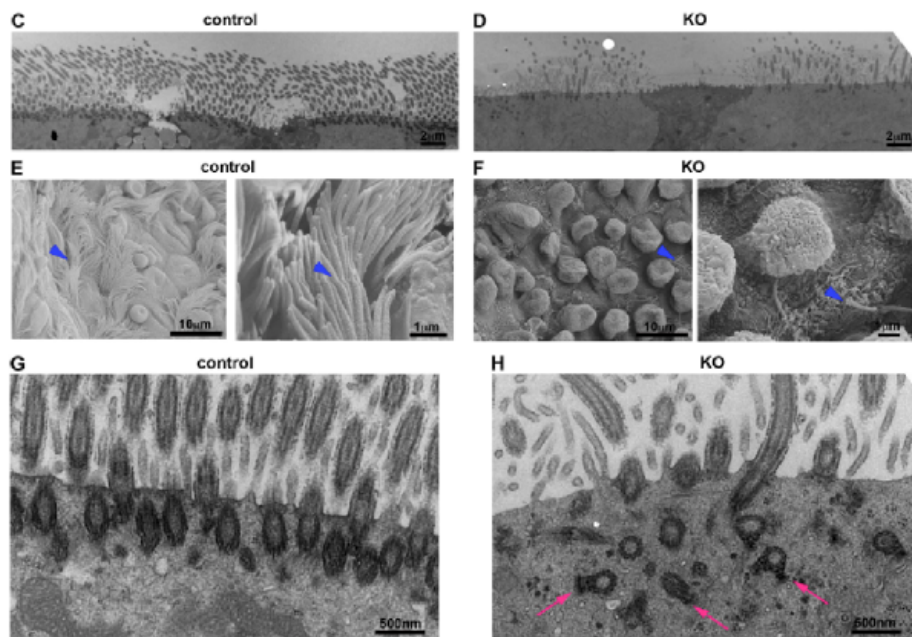
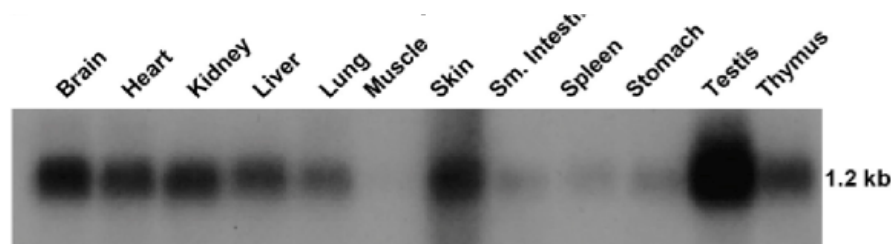
Bleach the embryos (final concentration 3% H₂O₂) by washing them in bleach under a hot lamp for 1-3 nights ART.

38. Store in 100% MeOH.

39. Photograph in PBS (follow the same 5-step hydration protocol as shown previously).

Fig S3. Cby is required for the normal number of motile cilia and basal body docking (Voronina et Al. 2009).

s3a) Northern Blot for a 120-KB transcript of Cby shows widespread expression but particularly in motile-ciliated cells (skin, testis, brain, kidney).



s3b) Voronina et Al. showed that in adult Cby ^{-/-} mouse nasal epithelium, the number of cilia are reduced (D vs. C and E vs. F.) and basal body docking is impaired (G vs. H).

In H, basal bodies are disorganized and a variety of IFT proteins are scattered around

the apical surface of the tracheal cells.