Knockdowns of axonemal proteins in Xenopus laevis cause unexpected transcriptional defects

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Knockdowns of axonemal proteins in *Xenopus laevis* cause unexpected transcriptional defects

An Undergraduate Honors Thesis

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Abstract

Members of the Klymkowsky and Winey labs have carried out morpholino experiments in *Xenopus laevis* resulting in transcriptional defects in ciliary genes. Whole embryo ISH has shown that when *EFHC1* is morphant, *Ntubb* mRNAs lose signal. In addition, *EFHC1* morpholino studies in animal caps show loss of cilia and multiple ciliary proteins. Moreover, qRT-PCR in animal caps has verified that knockdowns in *EFHC1* and *Cby* cause unexpected effects on transcription in wider transcription. This has compelled us to research *EFHC1* as a gene involved in ciliary transcription. I performed and here interpret wild-type in-situ for *Rfx2* and *TTC25*, two transcription-related factors required for ciliary transcription in Xenopus, and suggest further experiments.
Introduction

This undergraduate thesis presents novel data from the Klymkowsky and Winey labs for the vertebrate gene EFHC1 and its possible role in ciliary transcription. First I will review literature linking EFHC1 to ciliopathies, and then I will explain data possibly showing that EFHC1 is required for ciliary transcription.

A number of diseases, known as ciliopathies, have been linked to defects in the formation and function of cilia (Delgado-Escueta 1999, Murdoch and Copp 2010, Badano 2006). Cilia are axonemal organelles that project from the cell surface during G₀ or interphase cells (Ishikawa 2011). Motile cilia are long tubular organelles that extend from the apical surface of Eukaryotic cells. Motile cilia, which resemble flagella, usually have an axonemal 9+2 structure of microtubules, with two central microtubules that are connected to dynein to beat in a coordinated fashion with nearby motile cilia. Primary Cilia, which are found in vertebrates, are much shorter and have a simple 9+0 arrangement (Satir 2010). Ciliary structure, including basal bodies, the axoneme, and the transition zone are reviewed in Ishikawa et Al, 2011.

Primary Cilia are a feature of vertebrate cells that coordinate complex signaling transduction during interphase, or cell arrest, and are not well understood because they were long thought vestigial (Davenport 2005, Satir 2010). Mostly the primary cilium is required for signaling and chemosensation of fluid flow generated by the motile cilia. For example, primary cilia are required for a balance of Shh (ventralizing) and BMP (dorsalizing) signaling in the closing neural tube. Where Patched receptors (which inhibits pathway protein Smoothened) are lost in the neural tube cilia, the uninhibited Shh pathway causes ventralization of the NT and the posterior neural tube fails to form a lateral hinge point (Murdoch and Copp 2010). Spina bifida,
anencephaly, and craniorachischisis can result from increasing severity of the open neural tube. Renal cysts and polycystic kidney disease also result from signaling cilia failures (Badano 2006).

Motile cilia, in which the unexpected $EFHC1$ effects were found, are fluid-generating and motility organelles, conserved in Eukarya and necessary from neurulation throughout adulthood. The motile cilia are involved in functions from the oviduct shuttling of the egg and flagellar beating of the sperm cell (the flagellum is basically an elongated, solitary motile cilium), to generating left-right fluid flow in the embryonic node and clearing mucus from tracheal epithelium (Ishikawa 2011). Therefore, defects of motile cilia are linked to these functions: sinusitis, reproductive sterility, and situs inversus as a result of abnormal left-right body fluid flow are hallmarks of Primary Cilia Dyskinesia and the related Kartagener’s Syndrome (Davenport 2005, Badano 2006).

Over the past ten years the primary cilium has received renewed attention in developmental signaling studies (Davenport 2005), but motile cilia in model animals are revealing signaling functions previously thought to occur only in primary cilia. Motile cilia of the oviduct can sometimes contain ion channels necessary for signaling in addition to mechanical function (Badano 2006). In many cases, ciliopathies appear to be combinations of defects in motile and primary cilia (Murdoch and Copp, 2010).

One autosomally inherited ciliopathy is Juvenile Myoclonic Epilepsy, in which otherwise normal adolescents begin to experience myoclonic jerks, tonic-clonic seizures, and absence epilepsy (Dreifuss 1989, Delgado-Escueta 1984). 10-30% of epilepsies in hospital reports are linked to Juvenile Myoclonic Epilepsy (de Nijs 2013). Alcohol use, stress, fatigue, or sleep deprivation precipitate these symptoms, and some (5-8%) JME patients only experience myoclonus when stressed (Grisar 2010). Most experience epilepsies without stressors, especially when going to sleep.
JME has a genetic basis. JME is linked to missense, insertion, and deletion mutations in fifteen loci, including autosomal dominant mutations in genes such as \textit{GABRA1}, which is an ion channel, and a non-ion-channel \textit{EF-Hand-Containing 1, (EFHC1)} found in motile cilia (Suzuki 2012). \textit{EFHC1} is the only gene for which multiple affected families have been linked to the same mutations: Suzuki et Al. in 2004 found 21 patients in six unrelated Mexican families were linked to just five heterozygous missense mutations (table 1). Some studies dispute the linkage of \textit{GABRA1} and \textit{EFHC1} to JME (Ma 2006).

The role for \textit{EFHC1}, and it's protein EFHC1/Myoclonin/Rib-72, is complex since \textit{EFHC1} mutations are linked to ciliopathies other than JME, such as absence epilepsy (Delgado-Escueta 1999) and cryptogenic temporal lobe epilepsy (Suzuki 2009). Different authors have cited mutations in \textit{EFHC1} as underlying either 2, (Ma 2006), 5 (Léon 2009), 12 (Suzuki 2009), or 25% (Grisar 2010) of generalized idiopathic epilepsies, which are disorders with a strong genetic basis for which the patient has an apparently normal brain but has seizures.

The function of \textit{EFHC1} is unknown in part because it has three domains of unknown function (figure 1). The gene encodes an N-terminal 45-amino-acid region with an unconventional microtubule-binding site that associates with \(\alpha\)-tubulin at axons (de Nijs 2006). The microtubule-binding site is unique to \textit{EFHC1} and has an unknown function as it differs from canonical MAPs. The second region is a string of three tandem DM10 dom. DM10 domains are found singly in nucleoside diphosphate kinases, which bind NDP’s and convert them to NTP’s; however the DM10 is found singly in canonical NDKs (Roymans 2000). The EF-hand domain is variable in vertebrates—the algae \textit{Chlamydomonas reinhardtii} and mice have two in tandem (Ikeda 2005); humans have one EF-Hand domain on \textit{EFHC1} and no EF-Hand on \textit{EFHC2}, with an additional short-splice form of just one DM10 domain (Suzuki 2004); and \textit{Xenopus laevis}
have none, only an EF-Hand-less *EFHC1* orthologue. Figure 1 illustrates what is known and
unknown about these domains.

1A) Ikeda et Al. (2003) found that *Chlamydomonas reinhardtii* axonemal-binding gene Rib-72 was
analogous to vertebrate EFHC genes, such as *EFHC1* in *Mus musculus* (*Mm EFHC2* is not shown). In *Mus*
and *Chlamydomonas*, *EFHC* homologs contain two EF-Hand calcium-binding domains each (Ikeda 2005).

1B) The EF-Hand domain of Myoclonin in trout was isolated by Kretsinger et Al. (1973) and is redrawn here by Grisar et Al. (2010). Then it was only known as a Calcium-binding protein. The closed conformation is drawn on the left with six interacting amino acids.

1C) The "Hand" of EF-Hand proteins is a 12-AA loop that connects two longer helices, which
changes ionic bonds to close around free Ca\(^{2+}\) ions (Grisar 2010). Residues 1, 3, 5, 7, 9, and 12 of the
"Hand" bind Calcium, and the protein can dimerize but binds Calcium weakly in this conformation (Murai 2008).
Professor Klymkowsky used image software to compare additional vertebrate EFHC proteins. EFHC1 is 640-aa long in humans. All mutations of EFHC1 known to cause JME in unrelated families are found within the first two DM10 domains (Suzuki 2004), which are common to all vertebrates examined. *Xenopus laevis*, which is tetraploid, has only one copy of EFHC1 and without the Ca²⁺-binding domain. One interesting feature of the DM10 domains is that they are more highly similar to the homologous domains of other vertebrates than they are to each other. In addition, these tandem domains strongly bind the axoneme in *Chlamydomonas* (Ikeda 2003) so they could act as NDK regulators (Grisar 2010).

Table 1. Five heterozygous mutations in EFHC1 were linked to JME in 21 members of six unrelated families. 320 control individuals had none of these mutations (Suzuki 2004).

<table>
<thead>
<tr>
<th>Heterozygous missn. mut.</th>
<th>Amino Acid Substitution</th>
<th>Domain of EFHC1 affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>685TC</td>
<td>F229L</td>
<td>DM10 domain 2</td>
</tr>
<tr>
<td>628GA</td>
<td>D210N</td>
<td>Interdomain</td>
</tr>
<tr>
<td>757GT</td>
<td>D253Y</td>
<td>DM10 domain 2</td>
</tr>
<tr>
<td>229CA (double mut. w/ 662GA)</td>
<td>P77T</td>
<td>N-terminal MTBD</td>
</tr>
<tr>
<td>662GA (double mut. w/ 229CA)</td>
<td>R221H</td>
<td>DM10 domain 2</td>
</tr>
</tbody>
</table>

The expression pattern of EFHC1 was revealed by a combination of antibody, in-situ, northern blot and RT-PCR experiments, reviewed in Supplementary figure 1. The authors (Suzuki et Al. 2004 and 2008, Ikeda et Al. 2003 and 2005, and Leon et Al. 2009) found that EFHC1 was expressed in high levels in motile ciliated cells as well as a few cortex cells without motile cilia. mRNA signal was detected sequentially by Northern blots by Suzuki et Al. 2004. These signals were validated in sperm flagella and tracheal epithelia (immunohistochemistry for Myoclonin1, performed by Ikeda et Al. (2005), testes, oviduct, kidneys, and lungs and trachea, (antibody by Suzuki 2008) and in the ventricles of the brain and a few cortex cells without motile cilia (Leon 2009) (Supplementary figure 1). RT-PCR also verified an ambiguous in-situ that
claimed to show $EFHC1$ was minimally expressed at low levels in non-ventricular areas of the brain in adulthood (Ikeda 2005). RT-PCR confirmed the finding that $EFHC1$ is expressed in low levels in many areas of the brain (Leon 2009). Having shown that $EFHC1$ was expressed in areas that could affect neurotransmitters, knockout experiments were used to further understand $EFHC1$ function.

de Nijs et al. (2006) created knockdowns of specific domains of $EFHC1$, showing that the N-terminus of the Myoclonin protein was required for localization to the axoneme, and through analysis of that N-terminus, named $EFHC1$/Myoclonin an unconventional MAP (de Nijs 2006). In their following publication, they created four knockouts of Mem-GFP-$EFHC1$ corresponding to the first four mutations which were described by Suzuki et al. in 2004, and injected those constructs into two different HeLa cell lines (de Nijs 2012). Those mutations, D210N, R221H, F229L and D253Y, did not affect how memGFP-$EFHC1$ localizes to the cytoplasm during interphase and the mitotic spindle during M phase, but they did experience mitotic defects such as monopolar spindles, congression abnormalities, and delayed microtubule elongation (de Nijs 2012). More tellingly for JME studies, when the constructs were electroporated into stage 14.5 mice, cortical projection neurons failed to migrate from the sub-ventricular zone to the radial zone. (These results are shown in depth at the conclusory Fig 11). De Nijs et al. have argued from these results that $EFHC1$ has an unexplained effect unique to neurons. Because of the effect of axon migration, study of $EFHC1$ has focused on axonal defects; however other experiments have linked $EFHC1$ mutants to ciliopathies rather than axon migration.

An important change in the context of $EFHC1$ was when it was shown to have effects on the ependymal motile cilia of mice. In 2009, Suzuki et al. created a viable, reproducing line of $EFHC1$ null (-/-) mice with normal expression of $EFHC2$. These mice survived to adulthood but
nevertheless experienced eight times the spontaneous myoclonus compared to wild-type littermates (supplementary video 1). Other than a lowered threshold for myoclonus by pentylenetetrazol, the line of EFHC1-depleted mice had brains like that of a patient with JME (their brains were analyzed by EEG and GABA levels). Notably, these mice had no mitotic defects, and had a normal ciliary structure, but with slower ciliary beating (supplementary videos 1, showing WT; and 2, showing EFHC1/- mice). The impaired motility of these ependymal cilia raised the possibility that EFHC1 may have an effect specific to motile cilia.

EFHC1 has so far been understood as an axonemal microtubule-binding protein necessary for migration of cortical precursors and mitotic spindle organization (Rosetto 2011, de Nijs 2012 and 2013, Fig 11). However, the finding that the gene is specifically expressed in cells with motile cilia, and that when impaired, those cilia beat slower, suggests that EFHC1 may have roles in motile ciliogenesis that complement its role in mitotic spindle organization. Further understanding of this gene may be necessary to alleviating myoclonic seizures in individuals with EFHC1 mutations (de Nijs 2013); moreover, our experiments may lead to greater understanding of ciliary transcription.

The Klymkowsky lab has begun to investigate EFHC1 in the context of ciliary transcription. The lab has most recently investigated Cby and Wnt8 interactions and ciliary assembly which is nearly published (Shi, Zhao, Galati, Winey, & Kymkowsky, submitted). The Klymkowsky lab, in cooperation with the Winey lab, has performed further experiments that characterize EFHC1’s involvement in ciliogenesis through morpholino knockdowns of its DM10 domains.

Studies in Xenopus
The Klymkowsky and Winey labs have been using a translation-blocking morpholino molecule which temporarily blocks the expression of EFHC1 in Xenopus embryos and whose effects are viewed in animal cap explants. The Xenopus multiciliated epidermis is an extremely practical model system for the study of motile cilia in vertebrates and is used by many labs (Werner 2011). Several lines of evidence have justified the use of frog epidermis to study motile cilia of other vertebrates: firstly, in both groups, Notch-Delta signaling establishes a plane of goblet cells and smaller secretory cells interspersed with cells containing dozens of motile cilia (Ishikawa 2011); secondly, the motile cilia of trachea and Xenopus epidermis beat in a coordinated, unidirectional manner to generate tissue-specific fluid flow (Ishikawa 2011); and most importantly, both tracheal and ventricular cilia of higher vertebrates and Xenopus epidermis are specified by Sox proteins (Fawcett and Klymkowsky, 2004) and the FoxJ1 transcription factor (Thomas 2010). For these genetic and functional similarities, Xenopus epidermis is a suitable model system for most motile cilia.

Xenopus is also notable because it has one copy of the EFHC1 gene. That means that, while the animal is tetraploid, each copy of its Chromosome 6 (where EFHC1 is found) has basically the same version of EFHC1. This means that EFHC1 morpholino, unlike for EFHC1-/- mice, can completely knock down EFHC mRNA translation in Xenopus. Xenopus, in the sense of having one EFHC gene, is a superior model for If studies of EFHC1.

Our lab has produced novel data in *Xenopus laevis* epidermis that suggests that, where no alternate EFHC genes are present, EFHC1 mutants have unexpected embryonic defects in expression of many ciliary genes.
**Preliminary Results**

Recently the Klymkowsky lab has performed knockdowns of *EFHC1* by antisense morpholino for multiple DM10 domains and found unusual transcriptional defects (Shi, Zhao, and Klymkowsky, in preparation). Morpholinos temporarily block the antisense mRNA from translation downstream of where the molecule hybridizes; then the embryos are fixed in formaldehyde, preserving the temporary knockdown effect. Dr. Jianli Shi injected embryos with *EFHC1* full-morpholino and found decrease in *Ntubb*, which encodes Neuro-β-Tubulin, the GDP-binding structural subunit of microtubules (figure 2). Figures from Klymkowsky lab data are adapted from pictures by Dr. Shi and were shared by personal correspondence with Professor Klymkowsky and Dr. Shi. In each figure I indicate any prospective publications which are in preparation.
Figure 2. In Xenopus laevis, knockdown of EFHC1 reduces expression of Ntubb (Shi, Zhao, and Klymkowsky, in preparation). A-E. The gastrocoel roof plates of living stage 18 embryos were coinjected with control morpholino (A) or EFHC1 morpholino (B-E) as well as a separate strand of mRNA encoding nonspecific enhanced expression of mCherry, a red marker (shown by arrows). The embryos were fixed in the morphant state and hybridized for Ntubb, the mRNA encoding Neuro-β-Tubulin. Neuro-β-Tubulin is the tubulin subunit found exclusively in neurons in Xenopus (Moody 1996).

2A) In the Xenopus late neurula, cilia are transiently expressed in the gastrocoel roof plate (anterior folds), neural tube (posterior paired lines) and multiciliated epidermis (discrete lateral cells) during development. The neural folds continue to express Ntubb, such as in the axons of neurons, while the epidermal cilia are converted to mucus-secreting goblet cells in adulthood (Nishikawa 1992). Control MO is a short, modified DNA oligo marked with mCherry but which does not block any mRNAs.

2B-E) Neurulae were injected with EFHC1 MO before fixation; Ntubb expression is reduced in the mCherry-expressing areas where EFHC1 is morphant. Notice Ntubb is expressed normally where mCherry protein is less visible (D, E).

2F) A stage 35 in-situ hybridization for EFHC1 shows expression in multiciliated epidermis, the otic vesicle, and neural crest derived cells (Shi, Zhao, and Klymkowsky, In Preparation). This in-situ was actually the first experiment performed and it shows that EFHC1 in Xenopus is expressed in the same locations as it is in other vertebrates like mice. The Otic vesicle is the future ear; neural crest derived cells will become the trachea. Not indicated in 2F, signal is also seen in the pronephros, which will become the kidneys, and the forebrain which will contain ependymal motile cilia.

Figure 2 shows that EFHC1 has a novel and uncharacterized effect: EFHC1 MO knockdowns (2B-E) show massive downregulation of Ntubb in normally multiciliated cells. EFHC1 effects on Ntubb indicate effects for all epithelia with motile cilia. Xenopus gastrula and neurula epidermal cells are covered with dozens of motile cilia that generate a left-to-right fluid flow and later are required for neuroblast migration (Werner 2011). The stage 35 Xenopus also shows expression in multiciliated epidermis and motile-ciliated cells including the otic vesicle (which will become the ear) and the pronephros (motile cilia are used in the liver for fluid flow.) Therefore Xenopus EFHC1 is expressed in analogous structures to those of other vertebrates (Supplemental figure 1).
The *Ntubb* knockouts do not establish whether *EFHC1* acts directly on their transcription. Cilia have complex inductive relationships with which the complete cilium is required to maintain expression of structural genes, so the knockdown of *Ntubb* prompted further study of *EFHC1*’s upstream regulatory abilities. More generally, the lab wanted to see if cilia were reduced in morphants.

*EFHC1* morpholino was coinjected with an mRNA for enhanced membrane-bound GFP into neurula-stage embryos (animal caps shown in figure 3). *EFHC1* MO reduced the surface area of multiciliated cells, as shown by reduced expression of membrane-bound GFP at the coinjected cells (A vs. E). Cilia greatly increase the surface area of the plasma membrane of a multiciliated cell, so the effect in E indicates a rather dramatic loss of cilia.

MO reduced immunostaining of Centrin2 protein (3B vs. 3F), a small, pericentriolar Ca$^{2+}$-binding protein conserved in Eukarya and required for replication of mammalian centrioles as showed by mitotic failure where it is knocked out (Salisbury 2002). Centrin is required for singly-ciliated cells (DeLaval 2011) but apparently not for multiciliated cells whose centrioles are assembled de novo (Dawe 2007) such as Xenopus epidermis. In these cells, the mother centriole is duplicated by another protein complex. Nonetheless, CETN was lost in most of the epidermal cells in these explants, indicating that EFHC1 is required for presence of the centriolar protein (Shi, Zhao, and Klymkowsky, in preparation).

In the middle panel, EFHC1 morphants also had reduced immunostaining of acetylated-α-tubulin antibody (3C vs. 3G). α-Tubulin is the complementary component of microtubules and supports the finding in 3E that number of cilia were reduced. In the overlap (3D, H), we see that the antibody signals (B and C or F and G) overlap with multiciliated cells (A or E); in the EFHC1 MO cells, either the coinjected cell did not receive significant amounts of morpholino with the GFP; or else the morpholino did not block EFHC1 in those few cells. 3I and
K show a defect in neural crest migration that I am not attempting to explain. The results of E-H show that EFHC1 MO revealed another unexpected transcriptional defect. Relevant to these studies, in most of the cells injected, there was a huge loss of multiciliated cell area (E, G). Finally among the preliminary data, RTPCR experiments were used to quantify the changes in transcription in still more ciliary proteins (figure 4).

Fig 3. EFHC1 morphant embryos experience smaller multiciliated cell area as well as abnormal immunostaining of Centrin2 and α-tubulin. Whole embryos were coinjected with a morpholino, a separate mRNA encoding enhanced membrane-bound GFP under a ubiquitous promoter such as UAS, and antibodies for two ciliary proteins. Top panels show the effect of a control MO and bottom panels the effect of EFHC1 MO. The embryos were fixed and ectodermal cells cut away for fluorescent visualizations, except I and K which affect a separate set of cells.

3A) Membrane-bound GFP fluoresces at all membranes in these embryos, and the animal caps which are cut away show multiciliated cells, which have high area of cell membrane, as bright green dots.

3B) Immunohistochemistry in that same cap explant shows Centrin2 protein, which is a pericentriolar protein that is required for centriole duplication (deLaval 2011), is highly expressed in multiciliated cells.

3C) Immunohistochemistry for acetylated α-tubulin shows the acetylated form of α-tubulin, which is present particularly in cilia, where microtubules are relatively stable compared to cytosolic microtubules. AαT shows higher background expression than CETN.

3D) Merged images show that memGFP, AαT, and CETN protein are present in the same population of multiciliate cells.
3E-K) EFHC1 MO was injected and the same immunohistochemistry was performed. Expression of membrane-bound-GFP, CETN, and AαT are all reduced in morphant cells; whether this is because number of cilia or size of cilia is affected is unknown. Cells with significant loss of GFP, CETN and AαT overlap (3H), showing that EFHC1 MO has a negative effect on both proteins.

The final experiment that demanded my study was a “semiquantitative” reverse transcriptase PCR in Xenopus animal cap (ectodermal explant) cells (figure 4).

Figure 4. knockdown of EFHC1 and Chibby have unexpected effects on transcription in animal caps.

4A) semi-quantitative RT-PCR of animal caps shows the RNA levels of Ntubb and Wnt8 respond to Cby and EFHC1 morpholino (Cby MO also negatively regulates ligand Noggin and it’s receptor BMP4, not shown.) (Shi, Zhao, Galati, Winey and Klymkowsky). Compared to control morpholino, both EFHC1 and Cby morphant embryos have increased Wnt8 transcription and decreased Ntubb. Wnt8 upregulation in knockouts suggests that EFHC1 and Cby might cooperate somehow in Cby-related Wnt inhibition, but it is not known whether signaling will be increased because of increased ligand.
4B) shows in-situ for EFHC1 in stage 35 embryos. The picture points out expression in the otic vesicle, neural crest derived cells of the jaw, and ciliated epidermis cells; it also expresses in forebrain (anterior head) and the pronephros (three central mesodermal cells), all of which are motile-ciliated.

4C) In stage 19 embryos, Cby is strongly expressed in the neural tube and GRP but not in the multiciliated epidermis.

4D) In stage 35 embryos, Cby is expressed in the otic vesicle, neural crest derived cells, pronephros, myotome, and an unknown population of cells that may be blood islands.

When sq-RT-PCR was performed on EFHC1 and Cby morphant embryos, the morpholino knockdowns had similar effects on mRNAs encoding the signaling ligand Wnt8. EFHC1 morphants had the same upregulatory effect as morphant Cby, a canonical Wnt inhibitor. Cby docks to the membrane near the cilium when inactive, but when activated is transported to the nucleus where it blocks β-catenin transduction molecule from TCF/LEF binding sites. Wnt signaling is involved in myriad functions such as axon guidance in neurons, cell polarity, and neural tube patterning. Wnt pathway effects indicate a still wider regulatory effect for the motile-ciliary-expressed EFHC1.

The combined experiments above have combined to suggest totally novel effects from EFHC1. Loss of EFHC1 gene, which is expressed in motile cilia of Xenopus and other vertebrates (i.e. the mouse), causes drastically different phenotypes depending on how the knockout is performed and whether it is measured in vivo or in-vitro. In the Suzuki null-EFHC1 experiments (2009), mice are viable and experience only occasional myoclonic jerks. However in Xenopus, morpholino causes a broad knockout of ciliary genes. The epistatic relationship of EFHC1 to the transcriptional axis of ciliogenesis is far from understood; understanding EFHC1 may shed light into many ciliopathies related to loss of cilia or ciliary beating.

My experiments sought to describe the effects of EFHC1 on the transcriptional events of ciliogenesis. Ciliogenesis is a complex differentiation process integrating multiple signaling
pathways (Wnt, FGF, Notch-Delta) into a program that includes orientation of centrioles toward an apical surface, assembly of basal bodies and basal feet, and establishment of intraflagellar transport networks (Ishikawa 2011). My investigation sought a master ciliary transcription factor with which to test EFHC1 morphants.

In vertebrates, the master ciliary transcriptional factor is DNA-binding-protein Regulatory Factor X-2 (Rfx2). The seven vertebrate Rfx homologs are related to C. elegans DAF-19, which is necessary for the transcription of nearly all ciliary-specific genes (Swoboda 2000). Chung et Al. performed morpholino and in-situ screens in Xenopus and found that Rfx’s 2-4 are related to ciliogenesis, and Rfx2 is required for normal regulation of practically every structural gene related to the axoneme or intraflagellar transport in Xenopus cilia (Chung 2012). Where Rfx2 is knocked down, genes downregulated include IFT 122, IFT 172, TTC25, and PitX2, a factor further downstream of TTC25 (Chung 2012). Chung et Al. furthermore showed that Rfx2 is required for differentiation, but not specification, as α-tubulin still localized to the correct general arrangement and density of multiciliated cells around the epidermis. TTC25 has been identified as an important transcription factor by in vivo screens (Hayes 2007), and those authors showed that in TTC25-deficient embryos, the neural tube failed to close due to aberrant Shh signaling (Hayes 2007). Chung et Al. verified that regulation by Rfx2 was responsible for Shh effects: In embryos morphant for Rfx2 in the neural tube, cilia failed to penetrate the neural tube lumen, and due to transcriptional downregulation of TTC25, Shh signaling was decreased and the NT remained open (Chung 2012). Meanwhile, the cilia were shortened by about 50%, corresponding to confocal imaging of α-tubulin fibers, which were also about 50% shorter. These effects were reversed by rescue RNAs. These and other knockdowns by morpholino showed that Rfx2 and it’s downstream factor TTC25 are widely required for ciliogenesis, but are not required for specification. Both Rfx2 and EFHC1 morphants are required for certain ciliary factors, but not for
specification (EFHC1-MO/α-tubulin shown in figure 4G, Rfx2 MO/α-tubulin shown in figure 9G).

My goal was to determine the epistatic relationship between these two genes, if a simple regulatory relationship could be found. I hope these experiments have proven a fruitful first step into the investigation of EFHC1’s control over ciliogenesis.

**Materials and Methods**

The Wallingford lab, in which Chung et Al. performed experiments, provided us with vectors containing Rfx2 and TTC25 coding DNA. These plasmids were subcloned in competent E. coli and purified. DIG-labeled RNA antisense probes were synthesized by Promega RNA Polymerase Kit from the T7 promoter and DIG was visualized by an anti-DIG antibody. Finally, background signal was reduced by a 3% bleach solution until experiments approached the signal shown in controls. The Klymkowsky lab provided me with training and in-situ hybridization materials, including wild type and mutant Xenopus embryos.

I performed in-situ hybridization protocol according to Dr. Shi’s methods which she taught me at the bench. This method resembles Steinbeisser’s protocol and can be accessed in supplemental figure 2.

Mutant embryos (Cby, EFHC1 whole-MO, and Type 2 MO, which targets the 2nd DM10 domain) were prepared by performing Rfx2 or TTC25 in-situ in the appropriate fixed morphant embryos. These embryos were already hybridized for a control (Twist, Engrailed2, and Ntubb, respectively) which I bleached to a minimum before I began my experiment. While these embryos having been used once do not affect expression of my genes, I bleached them to
minimize their signal in relation to the signals I was testing. In my results section I indicated the hybridization performed by Dr. Shi and then my hybridization.

Results

Here I report three wild-type in-situ, *Rfx2*, *TTC25* (figures 5 and 6) and a control experiment *Engrailed 2* which shows that my protocol works given a probe with known signal (figure 7). I compare my wild-type in-situ to the prior studies of Chung et. Al. (Figure 8). Based on the results from Chung et Al., I think my in-situs provide a baseline with which to compare transcriptional regulation of *Rfx2* and *TTC25*.

Figure 5. I performed in-situs for *Rfx2* in wild-type Xenopus embryos.  
5A-C) show embryos stained with a high amount of probe (>400 ng) stages approximately 30, 26, and 13.  
5D-F) show embryos stained with a low amount of probe (>200 n) stages 26, 30, and 13.  
5A) I observe signal in the neural tube and otic vesicle and in neural-crest derived cells. Chung et Al. showed five *Rfx* paralogs with signal in the neural tube, so the neural tube was not weighed much for the probe quality.  
5B) I observed stronger signal in ciliated structures but with higher background signal. *Rfx2* is not observed in the pronephros as in figure 9Af. This may be due to a temporal difference in expression.  
5D-F) lighter staining is seen and in E the last signal to be removed by the bleach process was in the forebrain. The forebrain may have cilia related to the GRP or to ventricular ependyma, which are ciliated.  
5F) One embryo had two distinctive epidermal cells with expression. However this was the only stage 18 embryo where an epidermis cell could be distinguished above the background (see high background in 5C). I did not investigate whether *Rfx2* was expressed in sectioned GRP.
Figure 6. I performed in-situs for TTC25 in wild-type Xenopus embryos.

6A-D) Embryos were stained with a high amount of probe (>400 ng TTC25) stages 29, 24, and 13. These are not hybridizations with two concentrations of probe, but a variability of hybridization due to my protocol. My in-situs show a high variability of expression so I show both extremes.

6A) TTC25 is expressed in similar tissues as Rfx2 (see figure 8e).

6C) This embryo will be preserved and sectioned to see if TTC25 is expressed in the GRP. The GRP is a highly characteristic area of expression.
Figure 7. I performed in-situ for *Engrailed* 2 in wild-type Xenopus to act as a positive control for my protocol. Dr. Shi has used an En-2 probe solution to hybridize the anterior neural plate successfully many times to test her in-situ protocols. In addition, the Xenopus community has determined these images are consistent with a strong in-situ signal (review the signals with the Xenbase genome browser, [http://www.xenbase.org/gene/showgene.do?method=displayGeneSummary&geneid=866462](http://www.xenbase.org/gene/showgene.do?method=displayGeneSummary&geneid=866462)). 7A shows a stage 26, B shows a stage 29, and C shows a stage 13 embryo. Importantly, this gene has been probed many times in Xenopus and these images match the community image, showing my in-situ protocol is valid given a good probe. These embryos also showed me when to stop bleaching, because this level of expression shows what the Xenopus community considers a positive signal. The embryos must be bleached as long as these embryos to remove nonspecific signal; but they cannot be bleached longer or the protocol is removing hybridized probe.

7A, B) *En*-2 is expressed in the anterior neural plate; there is nonspecific staining in A. The difference in expression between A and B shows that my protocol produces hybridizations of varying strengths given the same concentration of probe. Stage 13 embryos also experienced varying levels of hybridization (other embryos like C were mostly blank after bleaching, and are not shown).
Figure 9. ISH as performed by Chung et Al (the Wallingford lab). These embryos are hybridized with the same probe as I used, so any differences in my signals are due to my protocol.


9B) shows a stage 19 embryo with normal NT closure (a) and, with Rfx2 MO, incomplete closure, especially in the anterior (Bb). Open NT defects are associated with and/or caused by lack of Shh signaling (Murdoch and Copp 2010). These NT defects could be rescued by injection of 200pg (.2ng) of Rfx2 mRNA (Chung et Al. 2012).

9C) shows wild-type in-situs for TTC25. It is expressed in the neural tube (a, b, c) epidermal multiciliated cells (b, c), GRP (a) and otic vesicle and kidneys (E).

9D) shows \( \alpha \)-tubulin in-situs (a, b) and TTC25 insitus (c-h). \( \alpha \)-tubulin signal is found in the same number of cells (b) (but Chung et Al. does show that the cilia are shortened), showing that those cells are specified for ciliogenesis by upstream factors such as Notch-Delta signaling. TTC25 signal is reduced in multiciliated epidermis (Dc vs Dd), neural tube cells (De vs. Df) and gastrocoel roof plate (Dg vs. Dh). Chung et Al. verified that Rfx2 MO was responsible for the loss of expression by RT-PCR in animal caps: in Rfx2 morphants, TTC25 was downregulated but A-Tubulin expression was unaffected (RT-PCR not shown).
Figures 5-9 show that my protocol produces embryos with variable signal, but nevertheless one that corresponds to the signals found in the Wallingford lab (figure 9). My positive controls were performed exactly alongside Rfx2 and TTC25, showing that my protocol produces a signal where true hybridization occurs. I performed the same protocol in embryos without a probe and those embryos were completely white after bleaching (not shown).

The high variability in my signals is a shortcoming of this preliminary data. 6 to 8 embryos were hybridized for each gene, and some hybridized with probe were completely white after bleaching. A larger number of embryos per experiment could show a more representative signal. Nevertheless, it seems I showed a signal for the genes hybridized.

Figure 10 shows my preliminary data for EFHC1-full MO: in-situ for Rfx2, TTC25, and Engrailed-2 are compared. In each embryo, Dr. Shi coinjected EFHC1-full-MO with an mRNA encoding enhanced mCherry, then performed in-situ for En-2; this in-situ produces two distinct bands at the roof of the forebrain (10A), even where MO is injected (10B). These embryos show some background signal.

To use these embryos, I needed to reduce the background staining for En-2 because Rfx2 and TTC25 are both expressed in multiciliated epidermis (figure 9) where that background is found in 10A and 10B. I bleached the embryos, then performed my in-situs using the same protocol as figures 5-7. Figures 10C-N show the result of double in-situs, which were inconclusive.

These double-in-situ embryos became very worn during this second in-situ because I am inexperienced with handling such young embryos. As a result, I had to take pictures for En-2 (C and D), Rfx2 (E-H), and TTC25 (I-N) before bleaching was complete. Because embryos were not completely bleached (see signals in C and D, which should be reduced to two bands at the
GRP), I cannot say whether my in-situs for Rfx or TTC25 show that the gene is expressed. However these pictures do illustrate the use of a double in-situ in orienting an embryo of unknown expression. These in-situs do show the challenge of performing in-situ twice on a single embryo.

Figure 10. I carried out whole embryo ISH in previously-prepared EFHC1 full-morphant embryos. Each one of these embryos had already been stained with En-2 as shown in A. Some of those embryos were injected with EFHC1 morpholino linked to mCherry as in figure 2. The right three columns show three in-situs carried out in these embryos: En-2 (to duplicate the signal), Rfx2, and TTC25. In each row embryos with the least injected morpholino are arranged at the top and those on the row below have greater marking.

10 A, B) EFHC-1 morpholino does not affect transcription of En-2 carried out by Dr. Shi. En-2 marks the anterior neural plate and the morphants do not show any gross developmental defect. Dr. Shi provided me with about 10 embryos from this set, 10 embryos injected with EFHC1-Type 2 MO, and Cby morphant embryos, of which about ½ were injected with morpholino. I split these embryos into cohorts of three and
carried out in-situ for the other genes. Most of these embryos became unusable during the course of my protocol because the first in-situ made them fragile and their epidermis completely sloughed away during washes.

C, D) These embryos show a nonspecific signal around the ciliated epidermis, with the paired bands of the neural tube not evident; upon further bleaching the embryos became unusable. Because the double in-situ was not made as specific as the first in-situ, it calls into question the specificity of the other in-situs.

E-H) En-2/Rfx2 double insitus reveal some areas where Rfx and En-2 could be coexpressed; however the Rfx2 signal is not necessarily specific enough to make a conclusion. Further invalidating these in-situs is the massive amount of sloughed-off epidermis (F, G) lateral to the neural tube. This pattern of lateral cells coming apart seemed to trouble this in-situ.

I-N) En-2/TTC25 embryos experienced somewhat less epidermis sloughing, and in places it is tempting to say that TTC25 is coexpressed with the MO (such as K), or not coexpressed (J, N). However, because the characteristic En-2 signal cannot be seen around them, and because the control En-2/En-2 could not be fully bleached, the embryos don’t prove anything. Apparent effects on TTC25 in N and J are due to sloughed-off cells, revealing mCherry which underlies the nearby epidermis.

Discussion: is EFHC1 transcriptional?

I have investigated the effect of EFHC1 knockdowns on transcription of ciliary DNA-binding protein Rfx2 and a downstream factor, TTC25. Morphant stage 18 embryos were inconclusive by in-situ hybridization. I have not made any new discovery into EFHC1’s function. However I have learned strategies for necessary further studies.

Whether EFHC1 should affect Rfx2 is unknown but extremely significant. Should Rfx2 be regulated it would indicate an even wider effect by EFHC1, either by Wnt signaling defects or by an indirect transcriptional effect. Rfx2 expression in the Xenopus GRP (equivalent to the node) is specified by Notch-Delta signaling, which specifies early ciliogenesis for epidermal cells scattered in the pattern of mucus-secreting and goblet cells (Hayes 2007). However, while Notch-Delta signaling is a specifier of Rfx2 expression, Wnt signaling is required for proper
expression of FoxJ1, a transcription factor required for Rfx expression, in the node of Zebrafish (Caron 2012). Caron et Al. showed that when Wnt8 signaling was reduced in the Zebrafish Kupffer’s vesicle, FoxJ1 was downregulated, motile cilia were fewer, and L-R patterning was disrupted (which is consistent with Rfx2’s regulation of TTC25) (Chung 2012). Therefore, as Wnt affects FoxJ1 in subsets of motile cilia, it is plausible that increased Wnt signaling in mutant embryos would lead to upregulation of subsets of Rfx2. If no effect on Rfx2 is found, then the Wnt signaling effects we found are likely not involved with the earliest determination of ciliogenesis. Rather, experiments would focus on how EFHC1 causes the transcriptional knockouts already seen. Knockouts of TTC25 in these embryos would also be a meaningful result as it would show EFHC1 being required for a transcription factor. EFHC1 has not yet been shown to regulate transcription factors, but this is plausible as TTC25 localizes to ciliary axonemes (Hayes 2007).

Our current transcriptional defects do not conclude on whether EFHC1 regulates ciliary transcription. In Dr. Shi’s experiments, EFHC1 knockdown results in an upregulation of Wnt8 ligand, and under a simple epistatic relationship, it would upregulate FoxJ1 and Rfx2 in the nodal cilia. In turn, Rfx2 upregulation could result in an upregulation of ciliogenic factors (not seen) or an oversaturation of cells differentiated for ciliogenesis, resulting in loss of homeostasis and failure of ciliogenesis. Genetic interactions between transcription factors, signaling pathways, and ciliogenesis are complex and we can’t attempt to deduce them from the existing knockout interactions. A thorough in-situ experiment for Rfx2 and TTC25 in EFHC1-morphant embryos is needed to understand how EFHC1 influences ciliary transcription.
**EFHC1** is a possible Wnt interactant

Further studies on *EFHC1* could focus on thus far unexplained regulational effects on *Wnt8* paired with *Cby*. Professor Klymkowsky and I discussed the possibility that *EFHC1* and *Cby* somehow cooperate to facilitate Wnt inhibition. Chibby is a Wnt inhibitor in vertebrates (Takemaru 2003) that localizes to the membrane near cilia (Enjolras 2012). Activated Cby migrates to the nucleus where it competitively blocks nuclear β-catenin from binding T-cell factor and lymphoid-enhancer-factor (TCF/LEF) promoters in the nucleus (Takemaru 2003). It is then involved in exporting β-catenin from the nucleus where the signaling molecule is degraded by proteases (Lee 2008).

Interaction between EFHC1 and Chibby is more than a simple positive regulation as qRT-PCR in *Cby* morphants showed additional negative effects on *Noggin*, a ligand produced in the node, and *BMP4*, its receptor (Shi et. al, “Unexpected signaling functions of ciliary proteins in *Xenopus laevis*”). The Klymkowsky and Winey labs are continuing to experiment on these signaling functions.

*Cby* mutants are somewhat consistent with effects found in *EFHC1* mutants. *Cby* loss-of-function mice show impaired motile ciliary beating (Figure S3, Voronina 2009) but also fewer basal bodies that migrate to the apical surface. Perhaps a partial downregulation of Cby could be related to the phenotypes shown in the supplemental videos where there are normal numbers of cilia with abnormal beating. However studies have not yet established that *Cby* is coexpressed with *EFHC1* (Voronina has shown by northern blot that it is widely expressed in motile ciliated cells, including skin, but this has not been established in frogs). Dr. Shi’s in-situ hybridization for *Cby* shows signal in most neural ectoderm, but not ciliated epidermis in high levels (figure 4c). A Cby antibody can be found in Xenopus multiciliated epidermis, but the
antibody acts nonspecifically and unreliably (Klymkowsky, personal communication.) We can not yet verify that Cby is expressed there (antibodies not shown).

EFHC1 could have effects on ciliogenesis by interacting with genes like Cby. RT-PCR studies could investigate whether Cby is expressed at some level in the epidermis cells. An RT-PCR strategy was used by Leon et Al. (2010) to clone EFHC1 from cDNA from nonventricular sections of the brain (cortex, hippocampus, striatum, and cerebellum) when it could not be found at adequate levels by in-situ. If EFHC1 and Cby can be reverse transcribed from cDNA made from the same epidermal cells, they could be coexpressed when the basal body docks to the membrane (however, proteins made from two mRNAs that are present at the same time do not necessarily interact).

Coexpression would justify that we perform true regulation experiments on EFHC1 and Cby. For example, transfected FoxJ1-promoted-GFP-EFHC1 would upregulate EFHC1 in the node where it is already found. In those cells, increased expression of Cby signal or quantitative RTPCR for Cby in those cells could reveal a regulatory relationship. Again, Cby morphants have transcriptional effects on Noggin and BMP4 not shown in EFHC1 morphants, so there is probably some other gene interaction attenuating their relationship.

EFHC1 is an unconventional MAP, required for spindle pole assembly and cortical progenitor migration. Migration defects could explain phenotypes of EFHC1 +/- mice.

The Klymkowsky and Winey labs have begun to show how knockouts of EFHC1 create unexpected ciliary transcription in Xenopus. However, we should consider the possibility that EFHC1 does not widely regulate ciliogenesis: it may perform post-translational modifications of
axonemes which are involved in an as-yet undescribed feedback loop, or affect a transcription factor we haven’t considered.

In conclusion I will review the effects of an overexpression of mutant EFHC1 which simulates the heterozygous missense mutations discovered by Suzuki et Al., 2004. Perhaps the defects in neurons caused by minor mutations can provide an alternate perspective on EFHC1 on ciliogenesis.

de Nijs et Al. have pointed out that EFHC1 is found in low levels of non-motile-ciliated cells such as the striated layers of the brain. There, EFHC1 affects migration of neurons from the early SVZ to the choroid plexus (figure 11). They hypothesize that EFHC1 has some unknown effect on axon outgrowth. In such a case, the missense mutations described by Suzuki et Al. have subtle defects causing the axon to grow too slow to accommodate normal instability of microtubules (reviewed in Ishikawa 2011).
Figure 11. Missense-mutant-type-EFHC1 can localize to the mitotic spindle normally, but experiences defects in chromosome separation and cortical migration (de Nijs 2012).

11A) HeLa cells were electroporated with six forms of mutant-EFHC1-GFP: four of these mutants represented substitution mutations which Suzuki identified as being linked with familial JME, and two were highly common polymorphisms found in patients with JME but not linked to JME. The mutant allele EGFP-EFHC1(D253Y) (middle row of Aa and Ab) is representative of the typical mitotic effect of a mutant allele for EFHC1 linked to familial JME (the others being D210N, R221H, and F229L). Electroporated EGFP-EFHC1(I619L) (bottom row of Aa and Ab) shows effects roughly equal to that of the other widespread polymorphism tested, R159W.

Aa) The protein encoded by EGFP-EFHC1 construct shows normal localization of EFHC1 to the cytosol and centrosome during interphase, where EFHC1 is normally found in interphase. The mutant constructs (middle and bottom rows) show the same localization, including the extensive cellular skeleton of these cells.

Ab) EGFP-EFHC1 localizes to the mitotic spindle as the normal protein does (shown by antibodies in previous studies), and metaphase of mitosis is rather beautifully illustrated in the overlap picture. The mutants also show normal spindle pole orientation (Ab, middle and bottom rows) as the EGFP-EFHC1’s align with the spindle pole. Merged images make the spindle poles difficult to discern but the left columns clearly show normal alignment of spindle poles.

11B) Mutant EGFP-EFHC1 constructs cause a higher proportion of mitotic spindle defects. Defects include monopolar spindle, in which the spindle poles fail to separate; and congression abnormalities, in which regions of chromosomes are incorrectly sorted. 32% of spindles with EGFP-EFHC1 had spindle defects, while 65% transfected with EGFP-heterozygous-mutant-EFHC1 had defects. EFHC1 modified with common polymorphisms showed no significant increase in these spindle pole defects.

11C) mutant-allele EGFP-linked EFHC1 disrupts radial migration of neurons.

11Ca) mutant-construct-EFHC1s were electroporated into mouse, and visualized after three days when 75% of axons normally reach the choroid plexus lumen. Mice expressing EGFP-EFHC1-(F229L) had the fewest axons reaching the choroid plexus, but all EFHC1 constructs with a JME-linked mutant had comparable increased migration defects as compared to EGFP-control-EFHC1.

11Cb) quantifies the regions to which the cortical neurons have migrated after 3 days. Lines show the % of neuron axons that have reached the labeled zone. The type of EFHC1 mutation affects % of axons that reach the choroid plexus by up to 15% (D253Y vs R159W). In the rat, such EGFP-mutant-EFHC1 constructs cause even greater loss of migration (de Nijs 2013, not shown.)
de Nijs et Al. used the EGFP-EFHC1 mutant constructs to simulate the common dominant-negative heterozygous mutants of EFHC1 linked to JME. Based on the results of their dominant-negative EGFP-mutant-EFHC1, de Nijs et Al. proposed that common heterozygous EFHC1 mutations misregulate the cortical excitation-inhibition network of synapses (de Nijs 2012). In support of this, they observe that the cortical axons that do migrate have twisting leading processes, disrupting synapses. They propose that EFHC1 exercises fine control over microtubule stability, and the four mutations described by Suzuki et Al. disrupt the cortical migration of neurons. The stability of neurons in development, they argue, is a determinant of fluid flow generated by ventricular cilia as much as motile ciliary beating in the ventricles, and perhaps ciliary defects stem from the neuron defects.

Impaired synapse transmission in JME brains may be related to cortical migration in addition or instead of the effects of EFHC1 on motile cilia in all vertebrates. It is also possible that the type of EFHC1 mutation relates to what side of development the mutant protein affects. As de Nijs et Al. put it in a 2013 review, “several but not necessarily exclusive hypotheses” can be proposed to explain defects in ciliogenesis and cortical neuron migration. Wnt signaling has a role in both those processes, so to find a connection between EFHC1 and Wnt regulation would prove a great finding in understanding these seemingly unrelated phenotypes in mutants. The findings of de Nijs provide us with a null scenario in which EFHC1 is involved in the stability of microtubules, while the role in ciliogenesis is still unknown.

**EFHC1’s involvement in Ciliogenesis is still unknown**

Our studies so far have focused on whole knockouts of Xenopus EFHC1, and established that they knock out a wide swath of ciliary proteins. As I repeat these in-situs for Rfx2 and TTC25 and establish their relationship to EFHC1, another candidate gene for epistatic
studies would be FoxJ1, which, like Rfx2, is responsible for a wide range of transcription, including upregulation of Rfx2. FoxJ1 experiments in EFHC1 morphants would emphasize the effects FoxJ1 has on basal body assembly proteins rather than IFT proteins which require Rfx2 (Thomas 2010).

Morpholino experiments have proven an inexpensive and quick way to knockout whole gene expression. Perhaps in addition to epistatic tests by morpholino, the next step in understanding EFHC1 would involve targeted knockouts analogous to the ones found in familial JME (such as F229L, which causes the greatest number of neurons to fail to migrate). Analogous knockouts of EFHC1 may show attenuated effects on ciliary transcription, such as knockouts in specific regions of the Xenopus embryo where EFHC1 has different interactants.

This thesis has outlined some of the major effects of EFHC1 null-MO on ciliary proteins. We have considered mechanisms for the loss of tubulins among the DM10 domains and interactions between EFHC1 and Cby in Wnt inhibition. I have reviewed that while loss of function in EFHC1 causes unexpected ciliary transcriptional defects, its role in ciliary transcription is unknown.

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