Cullin4B/E3-ubiquitin ligase negatively regulates β-catenin

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β-catenin is the key transducer of Wingless-type MMTV integration site family member (Wnt) signalling, upregulation of which is the cause of cancer of the colon and other tissues. In the absence of Wnt signals, β-catenin is targeted to ubiquitin–proteasome-mediated degradation. Here we present the functional characterization of E3-ubiquitin ligase encoded by *cul4B*. RNAi-mediated knock-down of Cul4B in a mouse cell line C3H T10 (1/2) results in an increase in β-catenin levels. Loss-of-function mutation in *Drosophila cul4* also shows increased β-catenin/Armadillo levels in developing embryos and displays a characteristic naked-cuticle phenotype. Immunoprecipitation experiments suggest that Cul4B and β-catenin are part of a signal complex in *Drosophila*, mouse and human. These preliminary results suggest a conserved role for Cul4B in the regulation of β-catenin levels.

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1. Introduction

Wingless-type MMTV integration site family member (Wnt) signalling is one of the important signal transduction pathways regulating several events during growth and development, and is also implicated in a variety of cancers (reviewed in Polakis 1997). Stabilization of β-catenin, a highly oncogenic protein, is the key event in transduction of the Wnt signal (reviewed in Polakis 1999). Binding of Wnt to its cell-surface receptor of the frizzled family causes the activation of Dsh, a cytoplasmic protein, which is then recruited to the cell membrane. Activated Dsh antagonizes glycogen synthase kinase-3 (GSK-3) β activity to stabilize and increase the levels of cytoplasmic β-catenin. Subsequently, β-catenin forms a complex with T-cell factor (TCF)/lymphoid-enhancing factor (LEF) to transduce Wnt signalling. In the absence of a Wnt signal, adenomatous polyposis coli recruits β-catenin to the Axin/GSK-3β complex. FWD1 or βTrCP, members of the F-box protein family, binds to phosphorylated β-catenin resulting in its degradation by the ubiquitin and proteasome pathways (Aberle *et al* 1997; Orford *et al* 1997; Jiang and Struhl 1998; Cong *et al* 2003; Nakayama *et al* 2003).

βTrCP is a component of the Skp1, Cdc53/Cullin1, F-box protein (SCF) complex which mediates the ubiquitination of a large number of protein substrates, particularly cyclin E (CycE) (Dealy *et al* 1999; Wang *et al* 1999) and β-catenin (Jiang and Struhl 1998; Cong *et al* 2003; Nakayama *et al* 2003). Cullins are a superfamily of ubiquitin ligases which are implicated in the regulation of several cellular functions (for a review *see* Petroski and Deshaies 2005). Cullins associate with ring proteins through their C-terminal domain, whereas the N-terminal region recruits a wide variety of receptor proteins that confer substrate specificity. They are modified by NEDD8 ubiquitin-like proteins, which stimulate their ubiquitin ligase activity (Podust *et al* 2000). There are as many as six conserved members of the Cullin family of ubiquitin E3 ligases in mouse and human (Kiproes *et al* 1996; Deshaies 1999). Normally, a specific Cullin family member is associated with the SCF complex, which provides substrate specificity. Although the role of βTrCP in β-catenin degradation is unequivocal, the identity of Cullin-E3 ubiquitin ligase specific to this process is not clearly understood. Cullin1 (Cul1) was the first Cullin family member to be discovered (Kiproes *et al* 1996), and is required to regulate the cellular levels of CycE (Dealy

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Abbreviations used: CycE, cyclin E; GSK-3, glycogen synthase kinase-3; LEF, lymphoid enhancing factor; RT-PCR, reverse transcriptase-polymerase chain reaction; SCF, Skp1, Cdc53/Cullin1, F-box protein; TCF, T-cell factor; Wg, Wingless; Wnt, Wingless-type MMTV integration site family member

et al 1999; Wang *et al* 1999). It is generally believed that Cul1 is also involved in *β*-catenin degradation, by virtue of its association with *β*TrCP (for a review *see* Petroski and Deshaies 2005). However, there is no direct evidence to show that Cul1 is indeed the E3 ubiquitin ligase required for this process.

With the help of loss-of-function genetics experiments, we show that Cul4B function is essential for *β*-catenin degradation in both *Drosophila* and mouse. Immunoprecipitation experiments suggest that Cul4B and *β*-catenin are part of a signal complex in *Drosophila*, mouse and human. These results, although preliminary in nature, suggest that the function of Cul4B in targeting *β*-catenin for degradation is conserved.

2. Materials and methods

Cell culture, *Drosophila* genetics, molecular cloning and associated nucleic acid and protein techniques were performed as per the standard procedures. A *dcul4* mutant was maintained over a green fluorescent protein (GFP) balancer (*CyO.KrGFP*). Homozygous larvae were identified by the absence of GFP. Viable excision lines (presumably precise excisions) were generated by crossing a *cul-4*^{KG02900} P-insertion line into a transposase source. The following monoclonal antibodies were used in this study: anti-Cul4B (Tripathi *et al* 2005), anti-*β*-catenin (Pharmingen), anti-tubulin (Santacruz), and anti-Armadillo and anti-Wingless (Development Studies Hybridoma Bank, University of Iowa, USA). Anti-Cul4B antibodies are of two types; antibodies against the N-terminal (CBN) and antibodies against the C-terminal (CBC) regions of mouse Cul4B (Tripathi *et al* 2005).

Maintenance and heat shock-induced differentiation of PCC4 cells was as described earlier (Gachelin *et al* 1977; Bisht *et al* 1994; Batth *et al* 2001). Total RNA and/or proteins were isolated from cells differentiating for 48 h, unless mentioned otherwise. For reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of Cul4A, Cul4B and Cul1 transcripts, primers were designed to amplify the 3′ end of each transcript.

2.1 *RNAi*

RNAi primers used to knock down Cul4B were 5′GATCCT TGACGACGTTGGAGTGTCGTCGATGCGACGACACT CCAACGTCGTCAATTTTT3′ and its reverse complement. Primers used for Cul4A were 5′GATCCTTCTCCTGTAG TACAGACAGTCGATGCGACTGTCTGTACTACAGG AGAATGAAG3′ and its reverse complement. Synthetic oligos were annealed and cloned into an MU6 promoter-containing eukaryotic vector (U Bhadra, unpublished). The plasmid was used at a concentration of 1.6 μg/ml to transfect 50–60% confluent C3H T10 (1/2) cells. LipofectaminePlus (Invitrogen) was used as the transfection agent. Cells were harvested 60 h post-transfection, since they (for both Cul4A and Cul4B) started showing signs of cell death by 72 h. Levels of both Cul4A and Cul4B transcripts were examined in each experiment. RNAi-mediated depletion of Cul4A did not have any effect on Cul4B and the reverse was also true.

3. Results

In both mouse and human, two closely related proteins represent Cul4 (Cul4A and Cul4B), coded by two different genes (Kiproes *et al* 1996; Li *et al* 2002). Detailed molecular characterization of mouse *cul4A* has been reported by Li *et al* (2002), who have also generated *cul4A* knock-out mice, which die at very early embryonic stages. We have previously reported that differentiating embryonal carcinoma cells (PCC4) are associated with increased levels of Cul4A and Cul4B transcripts (Tripathi *et al* 2005). These transcripts were identified by differential display RT-PCR analysis that was carried out with RNA from undifferentiated and differentiated PCC4 cells. We have cloned, expressed and raised antibodies against the amino and carboxy terminal halves of Cul4B. We have observed increased levels of Cul4B as well as its translocation into the nucleus during the differentiation of PCC4 cells (Tripathi *et al* 2005).

3.1 *Knock-down of Cul4B results in increased* β*-catenin levels*

Undifferentiated PCC4 cells express high levels of *β*-catenin (figure 1A), which is comparable with the colon cancer cell line colo205 (data not shown). Interestingly, curcumin, which induces differentiation of PCC4 cells (Batth *et al* 2001), causes a decrease in the levels of *β*-catenin in the colon cancer cell line HCT-116 (Jaiswal *et al* 2002). Curcumin treatment of PCC4 cells consistently caused a decrease in *β*-catenin levels (data not shown). Heat shock-induced differentiation of PCC4 cells was also associated with a reduction in *β*-catenin levels (figure 1A). This observation, coupled with the observation that PCC4 cells differentiate into cells of endodermal lineage (Tripathi *et al* 2005), make PCC4 cells a good model system to study the regulation of *β*-catenin.

As differentiating PCC4 cells are associated with increased levels of Cul4B (Tripathi *et al* 2005) and decreased levels of *β*-catenin, we tested the functional correlation between the two. For loss-of-function studies we used the mouse C3H T10(1/2) cell line, which shows lower levels of *β*-catenin than PCC4 cells. Double-stranded RNAi-mediated knock-down of Cul4B function (figure 1B) in C3H T10 cells

showed a significant increase in the levels of *β*-catenin (figure 1D). In contrast, RNAi-mediated depletion of Cul4A (figure 1C) caused a marginal increase in *β*-catenin levels (figure 1E). This suggests a specific requirement for Cul4B in *β*-catenin regulation.

3.2 *Loss of Cul4 function in* Drosophila *results in upregulation of* β*-catenin levels*

In *Drosophila*, there is only one Cul4 (*dCul4*; Cytology 44B1) protein of 90 kDa, which is 65% identical to both

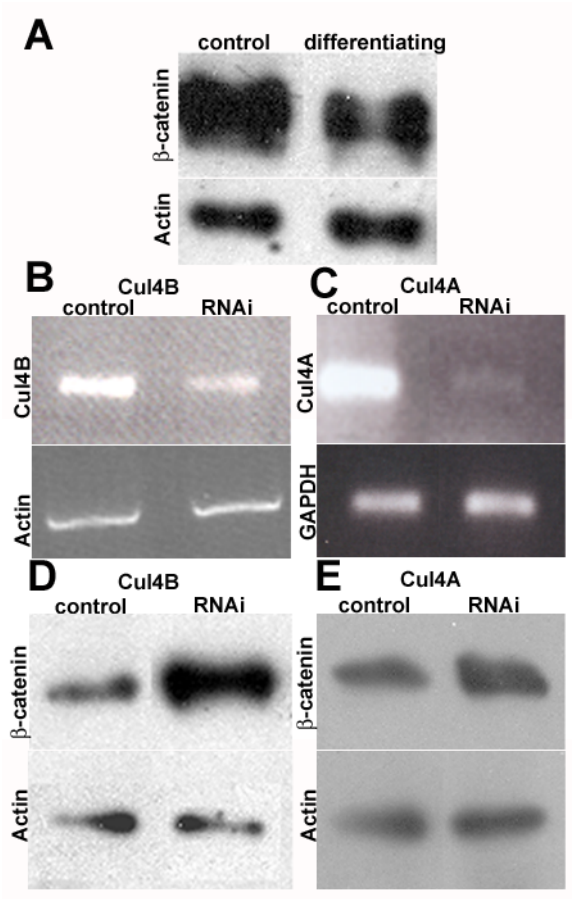

Figure 1. Negative regulation of *β*-catenin by Cul4B. (**A**) Western analyses of *β*-catenin levels in control and differentiating PCC4 cells. Note decrease in the levels of *β*-catenin during heat shock-induced differentiation of PCC4 cells. (**B**–**C**) RT-PCR analyses of Cul4B (**B**) and Cul4A (**C**) transcripts in C3H T10 cells: control and transfected with dsRNA (RNAi). Note that cells transfected with dsRNA against Cul4A or Cul4B show a decrease in the levels of their respective transcripts. We did not observe any change in Cul4B levels in cells depleted with Cul4A and vice versa (data not shown). (**D**–**E**) *β*-catenin levels in the same cells as in **(B)** and (**C**), respectively. Note the significant increase in the levels of *β*-catenin in cells depleted with Cul4B. Such an increase is not observed in cells depleted with Cul4A.

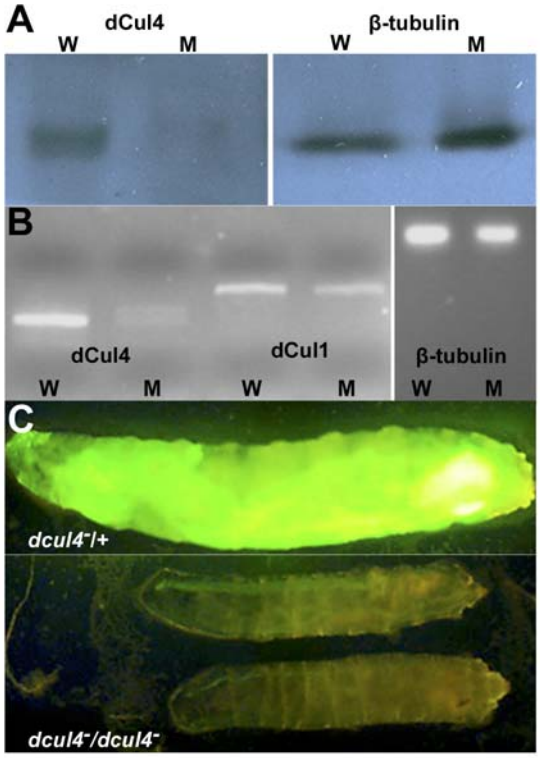

Figure 2. *dcul4* mutants are lethal for first instar larvae. **(A)** Western blot analysis with antibodies against mouse Cul4B of wild-type (W) and homozygous *Pcul4* (M) embryos. Mutant embryos are null to dCul4 protein. **(B)** RT-PCR analyses of dCul4, dCul1 and *β*-tubulin transcripts in wild-type (W) and homozygous *dcul4*$^{-}$ (M) embryos. Mutant embryos show severe reduction in dCul4 transcripts, whereas levels of dCul1 are unaffected. **(C)** Heterozygous and homozygous *dcul4*$^{-}$ larvae shown 3 days after hatching. Mutant embryos are identified by the absence of GFP, a marker on the balancer chromosome. Mutant larvae are very small compared with wild-type larvae. All larvae were imaged at the same magnification.

the Cul4A and Cul4B proteins of mouse and human. CBC antibodies cross-react with dCul4 (figure 2A) and hence the same was used in this study. dCul4 is ubiquitously expressed in all stages during embryonic development (data not shown; *see also* the Berkley *Drosophila* Gene expression database). We obtained *cul-4*^{KG02900}, a P-element insertion in dCul4, from the Berkley *Drosophila* Genome Project (*http://www.fruitfly.org*). It is inserted in the 5′ UTR at 203 bp upstream of the protein-coding region, which we re-confirmed by inverse PCR. Western-blot analysis using CBC antibodies suggested near-total reduction in dCul4 levels in homozygous mutant embryos (figure 2A). RT-PCR analysis suggested the absence of zygotic expression of dCul4 (figure 2B). Expression of dCul1 (Cytology: 43F1-2) is unaffected in these mutant embryos (figure 2B). Homozygous *dCul4* mutant larvae survive for 3 days, but do not moult or grow in size (figure 2C), a phenotype similar to loss of

Nedd8 in *Drosophila* (Ou *et al* 2002). In wild-type larvae, denticle belts mark the anterior compartments in the trunk segments (figure 3A). Loss of Wingless (Wg; *Drosophila* homologue of Wnt) signalling causes ectopic denticle belt formation, while gain of Wg signalling causes loss of denticle belt (also known as naked-cuticle) phenotypes (Lawrence 1992; Noordermeer *et al* 1992; Bejsovec and Wieschaus 1993). We observed the naked-cuticle phenotype in homozygous *dcul4*$^{-}$ larvae (figure 3B), which suggested gain-of-Wg function. Such a naked-cuticle phenotype was not observed in homozygous *cul1*^{Ex} larvae, a null allele of

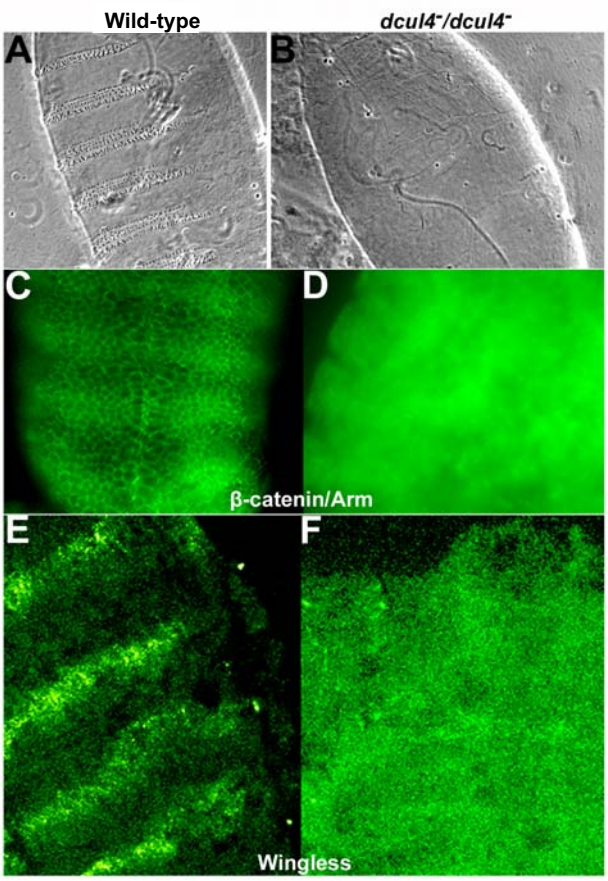

Figure 3. Negative regulation of armadillo by dCul4. **(A–B)** Ventral view of first instar wild-type **(A)** and homozygous *dcul4*$^{-}$ **(B)** larvae. The cuticle of mutant larvae is devoid of ventral denticle belts, a characteristic of overexpression of armadillo. **(C–D)** Wild-type and homozygous *dcul4*$^{-}$ embryos stained with anti-armadillo antibodies. **(E–F)** Wild-type and homozygous *dcul4*$^{-}$ embryos stained with anti-wingless antibodies. Both arm/*β*-catenin and wingless are ubiquitously expressed in mutant embryos and therefore the segmental expression pattern normally seen in wild-type embryos is lost. Heterozygous *dcul4*$^{-}$ embryos were identical to wild-type for both arm/*β*-catenin and wingless expression. (A) and (B) are imaged at the same magnification. (C) and (D) and (E) and (F) are imaged at the same magnification and also at the same camera exposure settings.

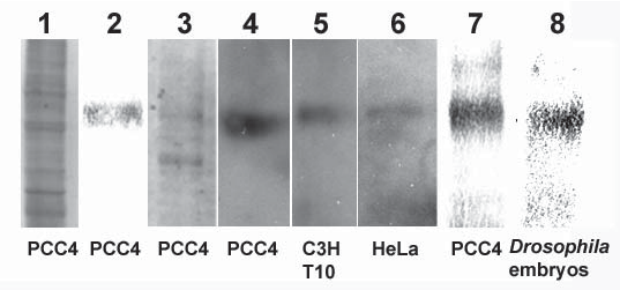

Figure 4. Physical interactions between Cul4B and *β*-catenin. Lane 1: Commasi-blue stained gel of the total cell lysate showing protein content. Lane 2: Western blot hybridization of total lysate with anti-*β*-catenin antibodies to show the position of *β*-catenin in the blot. Lane 3: Commasi-blue stained gel of the products immunoprecipitated with CBN antibodies. Note that 3–4 prominent bands are visible, one of which corresponds to *β*-catenin. Lanes 4–8: Immunoprecipitation with CBN **(**lanes 4–6**)** or CBC **(**lanes 7–8**)** antibodies of total lysate from mouse PCC4 cells, C3H T10 cells, human HeLa cells or wild-type *Drosophila* embryos followed by western blot analyses with anti-*β*-catenin (for mouse and human cells) or anti-armadillo (for *Drosophila* embryos) antibodies. Note that *β*-catenin is co-precipitated with Cul4B from all tissues. Mock immunoprecipitations with no primary antibody for all the cell lines and *Drosophila* embryos did not detect any bands when hybridized with anti-*β*-catenin antibodies (data not shown).

cul1 nor in any of the viable excision lines of *cul-4*^{KG02900} (data not shown). In wild-type embryos, Armadillo (*β*-catenin/Arm; *Drosophila* homologue of *β*-catenin) is found at higher levels in the cytoplasm of those cells wherein Wg is expressed (Peifer *et al* 1994) (figure 3C). Consistent with its naked-cuticle phenotype, homozygous *dcul4*$^{-}$ embryos showed uniformly high levels of *β*-catenin/Arm in both the anterior and posterior compartments of all segments (figure 3D). Since Wg itself is a target of *β*-catenin/Arm activity (Hooper 1994), Wg expression was also uniformly higher in both the anterior and posterior compartments of all segments in homozygous *dcul4*$^{-}$ embryos (figure 3F). These results are consistent with the function of dCul4 as a negative regulator of *β*-catenin/Arm.

3.3 *Cul4B and* β*-catenin are components of a single protein complex*

We then examined if Cul4B and *β*-catenin are part of the same protein complex and interact *in vivo*. We immunoprecipitated mouse Cul4B from PCC4 and C3H T10(1/2) cells, human Cul4B from HeLa cells and *Drosophila* dCul4 from wild-type embryos. Immunoprecipitates were subjected to western blot analyses with anti-*β*-catenin antibodies. In all the tissues examined, large amounts of *β*-catenin were found associated with Cul4B (figure 4), suggesting that the two are part of a single protein complex.

4. Discussion

Protein degradation is an important mechanism by which various cellular and developmental events are regulated. Often, a defective protein degradation machinery leads to disease conditions, particularly cancer. For example, upregulation of cytoplasmic levels of *β*-catenin causes cancer of the colon and other tissues. Colon cancer is one of the most studied cancers at the molecular level and several steps leading to the upregulation of *β*-catenin levels are well understood. Typically, it all begins with a mutation in the colon cancer gene *apc*. The product of the mutant *apc* gene fails to bind to *β*-catenin. Normally, in the absence of stabilizing Wnt signals, APC binds to *β*-catenin and carries the latter to the Axin–GSK–3*β* complex. *β*-catenin is phosphorylated by GSK-3*β*, which marks the former to ubiquitin proteasome-mediated degradation. This is brought about by a protein complex consisting of Skp1, F-box protein *β*TrCP and a member of the Cullin/E3 ubiquitin ligase family. In spite of a wealth of information being available on *β*-catenin regulation, the identity of the E3 ubiquitin ligase that ubiquitinates *β*-catenin is not known.

In this paper, we present a primary analysis of the function of mouse *cul4B* and its *Drosophila* homologue, *dcul4*. The preliminary results presented here suggest a critical role for Cul4B in mediating *β*-catenin degradation. Differentiation of embryonal carcinoma cells is associated with a significant increase in Cul4B (Tripathi *et al* 2005) and decrease in the levels of *β*-catenin (figure 1A). Consistent with this inverse relationship, RNAi-mediated depletion of Cul4B transcripts caused upregulation of *β*-catenin levels. RNAi experiments also suggest that Cul4A is probably not involved in this process, thereby indicating a specific requirement for Cul4B in *β*-catenin regulation. In *Drosophila* too, dCul4 is required to regulate *β*-catenin/Arm levels. Loss of *dcul4* causes defects in the formation of ventral denticle belts, a phenotype characteristic of gain-of-Wnt/Wg signalling. We also observed increased levels as well as ectopic expression of *β*-catenin/Arm and Wnt/Wg in *Drosophila* embryos mutant for *dcul4*. Finally, we observed that dCul4 in *Drosophila*, and Cul4B in mouse and human, are physically associated with *β*-catenin. These results suggest that the role of Cul4B/dCul4 in mediating *β*-catenin degradation is conserved.

A recent report suggests that human Cul4A and Cul4B are required to regulate CDT1 levels (Higa *et al* 2003). In *C. elegans* and *Drosophila*, Cul4 (both have only one *Cul4* gene) is known to regulate CDT1 levels (Higa *et al* 2003; Zhong *et al* 2003). In the light of our results, it is possible that Cul4B targets more than one protein for proteolytic degradation. However, we do not yet know if Cul4B is a part of the TrCP–SCF complex. Combinatorial interactions between different F-box proteins and Cullins may generate a large number of SCFs, each with a unique substrate specificity (Kitagawa *et al* 1999). Several questions remain unanswered. For example, what is the significance of Cul4B localization to both the nucleus and the cytoplasm, although *β*-catenin degradation is a cytoplasmic event? How is Cul4B activity regulated? How is substrate specificity achieved? Assuming that Cul4B is a part of the TrCP–SCF complex, is there a specific interaction between the F-box protein *β*TrCP and Cul4B to come together and target *β*-catenin for degradation? Further investigation in this area may provide satisfactory answers to these questions.

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