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Original Article

Furry is a component of the CCM3-GCKIII signaling pathway

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ABSTRACT

Aim: Mutations in 3 genes encoding proteins of the Cerebral Cavernous Malformations

(CCM) ternary complex, cause autosomal dominant cerebral vascular disease. Targets of CCM complex regulation have been identified; however, the molecular mechanisms connecting CCM3 to these downstream effectors remain elusive. We aim to determine the mechanism of CCM3 action by using a *Drosophila* model to elucidate the signaling pathway downstream of CCM3. Previously, we showed that CCM3 and its binding partner, Germinal Center Kinase 3, are required in tracheal terminal cells to prevent tube morphogenesis defects. Further, we established that GCKIII phosphorylates and directly activates a downstream kinase, Tricornered (*Drosophila* STK38/38L ortholog). Here we aim to test whether Tricornered-associated scaffolding protein, Furry, is required for CCM3-GCKIII signaling.

Methods: We utilized the FRT-FLP system to generate genetic mosaic *Drosophila* larvae and adults. Mitotic recombination was induced in embryos (trachea) or larvae (wing disc). The animals were heterozygous for the gene of interest (*ccm3* or *furry*), but after recombination, homozygous mutant daughter cells were produced. In addition, the GAL4-UAS system was used to express dominant negative GCKIII in wing disc cells. Mutant cells were analyzed by brightfield and/or fluorescent microscopy.

Results: We find that wing cells mutant for *ccm3,* or expressing dominant negative GCKIII, produce wing hair defects characteristic of mutations in *tricornered* and *furry*. Likewise, tracheal terminal cells mutant for *furry* produce tube dilation defects characteristic of cells mutant for *ccm3* or *GCKIII*.

Conclusion: CCM3 and GCKIII act upstream of Furry-Tricornered, suggesting the conservation from yeast of a Hippo-like signaling pathway that regulates morphogenesis. We speculate that some combination of Furry/Furrylike and STK38/38L are therefore likely to act downstream of CCM3 in endothelial cells.

Keywords: CCM3, GCKIII, NDR, STK38/38L, tube morphogenesis, planar cell polarity

INTRODUCTION

Cerebral cavernous malformations (CCMs) are common vascular defects found in the capillaries of the central nervous system (reviewed in Lampugnani et al. [1]). Familial CCM shows an autosomal dominant manner of inheritance, with variable expression and incomplete penetrance (reviewed in Riolo et al. [2]). Somatic loss of a second (Knudsonian 2 hit mechanism) CCM allele [3] is believed to result in localized vascular lesions in which grossly dilated, thin-walled capillaries lacking surrounding support cells undergo repeated hemorrhage leading to headaches, neurological deficits and stroke [4]. Lesions are believed to be largely clonally derived, containing few wild type endothelial cells [5] (although also see [6]). Mutations in three different genes cause disease and account for at least 80% of cases of familial CCM. These genes – *Krev interaction trapped protein 1* (*Krit1), Malcavernin*, and *Programmed Cell Death 10* (*PDCD10)* – are also known as *CCM1*, *CCM2* and *CCM3,* respectively. Generally, the CCM proteins have been thought to act as scaffolding for regulators of cytoskeletal remodeling and/or signaling pathways. The CCM proteins can physically interact, forming a ternary complex bridged by CCM2. The complex may be recruited to the plasma membrane through interaction with a putative transmembrane receptor protein, Heart of Glass [7], or by interaction with the Vascular endothelial growth factor receptor 2 (VEGFR-2) [8, 9]. The precise functions of the individual proteins, and of the various complexes, remain key subjects of inquiry. The role of CCM3 has been particularly controversial, as CCM3 is also found in other complexes [10] and has been suggested to operate in a CCM1- and CCM2-independent manner [11-15].

Consistent with the idea that CCM3 may act independently of CCM1 and CCM2, mutations in *CCM3* cause an earlier lethality in mice, and in humans, patients with *CCM3* mutations show a much earlier age of onset and a more severe course of disease [12, 16-18]. Likewise, patients with mutations in *CCM3*, but not *CCM1* or *CCM2*, develop

multiple meningiomas at a high frequency [17]. These differences may reflect a requirement for CCM3 in epithelia [19], or may hint at a distinct molecular function for CCM3 even in endothelial cells.

We have turned to the simple *Drosophila melanogaster* model system to dissect CCM3 function. Importantly, CCM3 is well conserved, but neither CCM1 nor CCM2 orthologs have been reported in flies. In prior work, we identified a requirement for CCM3 and GCKIII in tracheal terminal cell tube morphogenesis [20], and have since gone on to show that GCKIII directly activates the Nuclear Dbf2-related (NDR) kinase, Tricornered (NDR1, 2 in vertebrates, also known as STK38 and STK38L) by phosphorylation [21]. Altogether, our work shows that CCM3 is a novel component of an ancient kinase cascade conserved from yeast: the RAM (Regulation of Ace2 and Morphogenesis) pathway in budding yeast, and the MOR (Morphogenesis Orb6 network) pathway in *Schizosacharomyces pombe* [22].

In yeast and flies, NDR kinase signaling typically requires co-factors including Mob (mps one binder) and Furry family members [23]. Four Mob family members have been identified (Mats, Mob2, Mob3 and Mob4) in flies, and single Furry protein (Tao3p in budding yeast and Mor2p in fission yeast). So far work on the invertebrate *fry* genes indicate essential roles in Trc signaling, with known requirements in the generation of actin-based cellular protrusions (wing hairs and bristles [24-30]), in the control of dendritic branching and tiling in dendritic arborization neurons [31-33], and in follicle cell polarity [34]. The vertebrate orthologs of Fry (FRY and FRYL) have been implicated in several processes [23], including tubulogenesis in the kidney [35, 36], but have not yet been linked to vascular biology.

METHODS

Wings. Young adult flies (newly eclosed up to 1 day old) were anesthetized on CO_2 pads and then transferred into 100% ethanol in glass dissection dishes. Using

112 microdissection spring scissors and Dumont forceps (Fine Science Tools, Foster, CA,
113 USA), the wings were clipped off at their attachment site. Wings were transferred to a
114 drop of Euparal (BioQuip, 6372A), and a coverslip was applied. Weights were placed
115 on the coverslip to flatten the specimens, and slides were allowed to dry at room
116 temperature for 1 day or more before imaging. Images were captured at the wing
117 margin and just distal to the cross-vein connecting wing vein 3 and 4. For *ccm3*
118 mosaic wings, virgin FRT^{82B} *ccm3*/TM6B flies were crossed with *y, w,* $hsFLP^{122}$;
119 FRT^{82B} males, and the progeny (embryos and larvae) were subjected to heat shocks
120 (38.5 ^{o}C) for 1 – 2 hrs starting at 6 hrs after egg lay (a.e.l.). Heat shocks were
121 repeated every day through the end of the 3rd larval instar. Heat shock induction of
122 Flipase (FLP), a site-specific recombinase, resulted in mitotic recombination between
123 the homologous chromosomes at a centromere proximal FLP Recombinase Target (FRT)
124 site (FRT^{82B}). As a consequence, genetic mosaic animals were produced with most
125 cells being heterozygous, but with clones of homozyougs wild type and homozygous
126 mutant cells. Mosaic adults were easily recognized by eye color mosaicism, as well
127 as by an unevenness of the wing surfaces (mosaic wings appeared somewhat crinkled
128 rather than the wild type flat appearance that characterized the wings of their TM6B
129 siblings). For $GckIII_{T167A}$ wing analysis, the wing specific *nub*GAL4 driver flies were
130 crossed to the UAS-$GckIII_{T167A}{}^{F2}$ flies (dominant negative, non-phosphorylatable
131 GCKIII [21]).
132
133 Mosaic analysis of larval trachea. To test the cell autonomous requirement for *furry* and
134 *Mo25* in tracheal cells we generated genetic mosaic animals using FLP-FRT
135 approaches [37, 38]. The alleles fry^{O31}, fry^{O41} and fry^{O98} were gifts from Dr. Sally
136 Horne-Badovinac and encode truncated Fry proteins (Q1008term, W394term and
137 G666term, respectively). The fry^{1} allele [23] carries a 1 bp deletion causing a
138 frame-shift after aa 403. Lastly, the fry^{s308} allele comes from an EMS/X-ray screen for
139 modifiers of *sina*GMR.PN eye phenotype [40]. Except for fry^{s308}, all *fry* alleles were
140 induced on chromosomes carrying FRT^{80B}. For these alleles, mosaic larvae were
141 generated using the MARCM strategy. For fry^{s308}, we generated recombinant

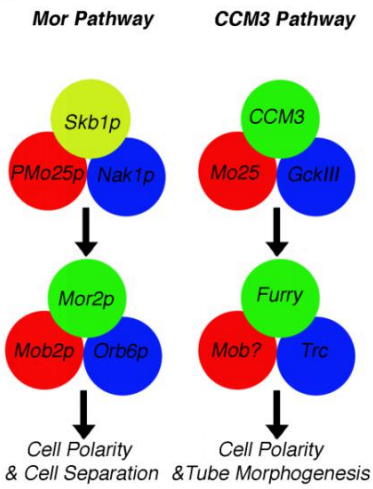
chromosomes carrying both the *fry* mutation and FRT^{2A}, and generated mosaic larvae using a MARCM-related approach we had previously developed, substituting a GFP RNAi transgene for the *Tubulin*-GAL80 transgene. For *Mo25*, the D8-2 allele was used [39]. Except for analysis of fry^{s308}, virgins of the genotype y, w, hsFLP122; *btl*-GAL4, UAS-GFP, UAS-DsRED2nls; *Tub*-GAL80 FRT80B were crossed to males carrying fry mutant alleles on FRT80B chromosomes in trans to TM3Sb, Twist>GFP. For the fry^{s308} analysis, virgins of the genotype y, w, hsFLP122; btlGAL4, UAS-GFP, UAS-DsRED2nls; UAS-GFP RNAi FRT2A were crossed to males of the genotype fry^{s308} FRT2A/TM3, Sb. Crosses were established with 40 virgins and 20 – 40 males. After 4 hrs at 25 °C, adults were transferred to fresh vials, and the 0-4 hrs a.e.l. embryos were subjected to 45 – 60 minute heat shock. The embryos were then cultured for an additional 5 days at 25 °C and analyzed at the third larval instar. Mosaic larvae were identified based on the presence of individual GFP expressing cells in the tracheal system (marking the homozygous mutant clones). The mosaic larvae were then heat killed (~ 10 seconds at 70 °C) in a drop of 50% glycerol on a slide; a cover slip was added and fluorescent microscopy was used to identify mosaic animals. Mutant terminal cells were analyzed by brightfield microscopy to score the presence and shape of gas-filled tubes. Cells were also analyzed by fluorescent microscopy, with the black space in GFP labeled cells revealing tube lumens, including the lumens of tubes that were not gas-filled (and thus not detectable by brightfield microscopy). Larvae were examined using 20X and 40X objectives on a Leica compound fluorescent microscope. Z-stacks were captured for each GFP positive terminal cell using Leica software. Unidentified images were then scored independently for the presence of transition zone tube dilations and other defects. In some instances, the position of the terminal cell within the specimen did not allow analysis, these cells were excluded from counts.

Microscopy. Larvae were imaged using direct fluorescence and Brightfield optics using a Leica DM5500 B upright widefield epifluorescence microscope (Leica

Microsystems). Images were acquired using a Leica DFC360FX camera. Z-stacks were captured and processed by deconvolution using Leica Advanced Fluorescence Application Suite (Leica Microsystems). For wing hairs, wings mounted in Euparal were examined with brightfield optics using a Leica DM6000 inverted microscope, and images were captured using a Hammamatsu Orca-R2 Digital CCD camera (C10600, Hamamatsu Photonics).

RESULTS

We previously identified a requirement for the CCM3-GCKIII signaling pathway in the *Drosophila* tracheal system [20]. Importantly the CCM3-GCKIII tube morphogenesis program shares common components with an orthologous pathway in the human vascular system (reviewed in Riolo et al. [2]), that is partially conserved from yeast (**Figure 1**). Prior to our work, Tricornered (Trc), the most downstream kinase in the cascade, was best known for its role in the morphogenesis of other tissues [28, 31, 40]. We decided to test whether regulation of Trc by CCM3-GCKIII was tissue-specific, or instead might be a general feature of Trc regulation. To do so, we turned to the *Drosophila* wing, a system widely used for studying developmental signaling pathways and planar cell polarity [41-46].



	Paralogs	
S. pombe	*D. melanogaster*	*H. sapiens*
None	CCM3	CCM3
PMo25p	Mo25	CAB39,39L
Nak1p	GckIII	STK24,25,26
Mor2p	Furry	FRY, FRYL
Mob2p	Mats,Mob2,3,4	Mob1A,B,2,3A,3B,3C,4
Orb6p	Trc	STK38,38L

Figure 1: CCM3 is a novel regulator of a conserved Hippo-like signaling pathway. The fission yeast Morphogenesis (Mor) pathway is defined by a short

kinase cascade consisting of a Sterile20-like kinase (Nak1p) that phosphorylates and activates an NDR kinase (Orb6p) which in turn phosphorylates effectors required for cell polarity and separation [47]. Many of the proteins in this pathway are conserved among eukaryotes, with CCM3 representing a novel scaffolding protein, not present in yeast, for the upstream kinase complex. CCM3 binds directly to GCKIII in human, zebrafish and flies [12, 48, 49]. Likewise, Mo25 can bind directly with Sterile20-like kinases including GCKIII family members[50]. Direct interactions between CCM3 and Mo25 have yet to be reported.

CCM3-GCKIII pathway regulates wing hair morphogenesis.

In flies, wing epithelia possess planar polarity that is easily read out in each cell by the position and orientation of actin-based cellular protrusions called wing hairs. In wild type wing epithelial cells, a single hair that tapers to a point extends from the posterior vertex of the cell, points distally, and is aligned with the hairs of neighboring cells (**Figure 2A**). In *tricornered* and *furry* mutant cells, the organization of the actin-based hairs appears to be disrupted such that the hairs split, giving rise to multiple hairs (**Figure 2B, E**) and/or hairs with split ends [24, 28]. Although prior studies suggest no role for Mo25 in wing hair morphogenesis [30], we decided to determine if disruption of *ccm3* and *GckIII* activity would give rise to wing hair defects characteristic of *trc-fry* [26, 27], we examined wings expressing a dominant negative GCKIII isoform (**Figure 2C, F**, Gck_{T167A} [21]) , as well as *ccm3* mosaic wings (**Figure 2G**). In both cases, we found that the affected wing cells developed multiple wing hairs with disruption of hair orientation.

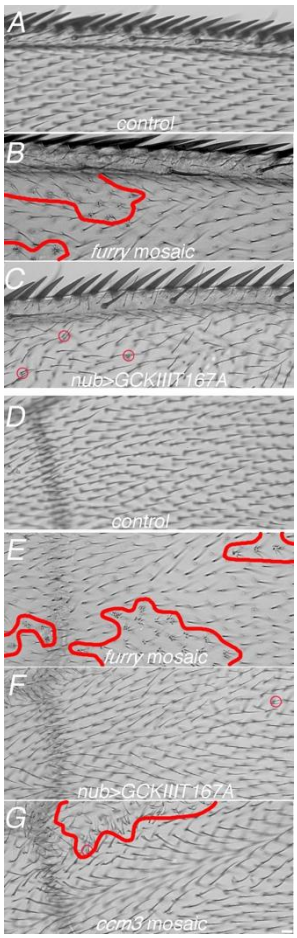


Figure 2: Like mutations in *furry*, loss of CCM3 or GCKIII function cause wing hair number and polarity defects. A. Anterior wing margin of a control wild type (OR-R) fly is shown. Note that individual wing hairs are aligned, and point towards the distal wing tip (to the right). In flies mosaic for *furry* (border between cells in the mutant clone and wild type cells outlined in red) (B), or expressing a dominant negative (non-phophorylatable) GCKIII isoform ($GCKIII_{T167A}$) (C), orientation of the wing hairs is disrupted and (red circles) two or more wing hairs per cell may be produced. D. Wild type wing hairs in the area immediately proximal to the crossvein connecting veins 3 and 4 (dark pigmentation running vertically on the left of image) are shown. Note the presence of single wing hairs per cell that are oriented towards the distal wing tip (right). In *furry* clones (E) (clone borders marked in red), or wing cells expressing a dominant negative (non-phophorylatable) GCKIII isoform (F, $GCKIII_{T167A}$), orientation of the wing hairs is disrupted and (red circles) two or more wing hairs per cell are produced. G. In wings that are mosaic for loss of *ccm3* function (clone outlined in red), mutant cells produce multiple wing hairs and display altered orientation. We note that in addition to the number of wing hairs per cell, mutations in the CCM3/GCKIII-Trc/Fry pathway may also affect planar cell polarity such that the orientation of the hairs appear perturbed. Scale bar in G (for A-G) = 10 microns.

Furry is required in tracheal terminal cell tube morphogenesis

Furry has been identified genetically and biochemically as a partner for Trc [24, 26, 27], and has been shown to function together with Trc in the shaping of actin-based cellular projections (wing hairs, etc.) in epithelial cells, as well as the polarized deposition of basement membrane [23]. In neurons, Fry has been shown to function with Trc in the

morphogenesis of dendrites [31]. A role for Fry in tubulogenesis has not been examined, although *fryl* mutants in mice have been reported to have kidney defects attributed to a role in the regulation of a microRNA [35, 36]. Because Furry appears to be required in all other contexts where Trc function has been described as necessary for morphogenesis, we tested if *furry* was also required in the tracheal system. For this analysis we characterized the tracheal terminal cell phenotype of 5 independent mutant alleles of *furry*, derived from 3 different genetic screens [25, 34, 51].

As has been the case for other genes in the pathway (*tao, ccm3, GckII*[20, 21]*I;* **Figure 3 A,B**), and is also true for *Mo25* (**Figure 3 C**) zygotic loss of *fry* appeared to uniquely disrupt tube morphogenesis in terminal cells, but not other tracheal cell subtypes. In homozygous *fry* terminal cells (**Figure 3 D**), we observed a range of phenotypes, running from cells with tubes indistinguishable from wild type, to cells with multiple transition zone dilations. The penetrance of the dilation defects varied from allele to allele, with the fry^{s308} allele showing the most penetrant phenotype (~80%, 25/31 cells showing clear transition zone dilations). In addition to transition zone tube dilations, less prominent tube dilations throughout the terminal cells were also observed, consistent with our prior results for other pathway members. In addition, we observed gas-filling defects and melanization of the transition zone dilation – these defects were less common, with lack of gas-filling observed in about 5% of mutant cells and melanization in about 2% of mutant cells (n = 183 cells scored). All of these defects were also described for *tricornered* mutant terminal cells[21], and none were observed in wild type control larvae, or in the heterozygous terminal cells in *fry* mosaic larvae (n = 50 and 50, respectively). The incomplete penetrance of the *fry* phenotype may indicate a less stringent requirement for *fry* than for *GckIII* and *trc*, or may reflect the presence of maternally supplied *fry* mRNA and/or protein.

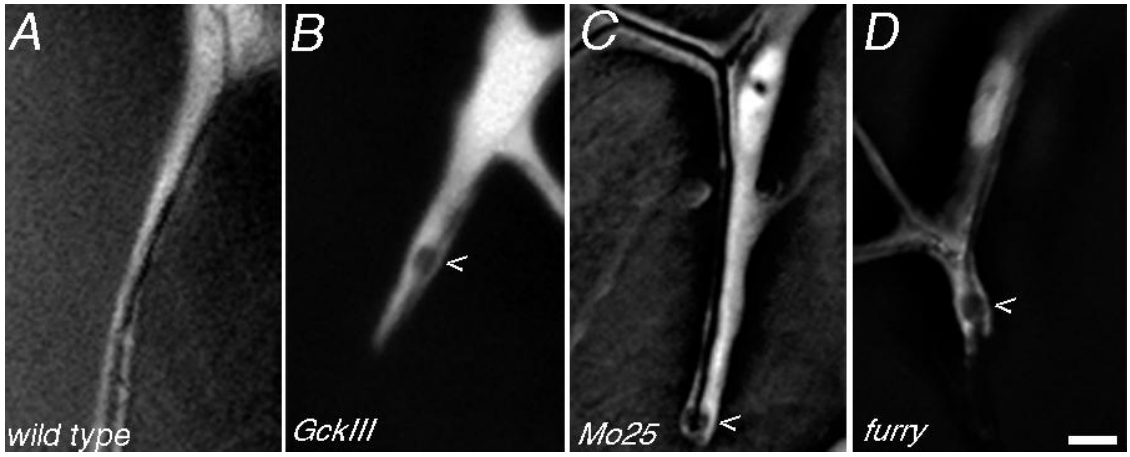


Figure 3: Like mutations in known CCM3-GCKIII signaling pathway components, *furry* and *Mo25* loss of function result in tube dilations in tracheal terminal cells. A. The most proximal part of a wild type control terminal cell clone (GFP positive labeling of homozygous cells) is shown, depicting the portion of the terminal cell that extends from its intercellular junction with a neighboring stalk cell, at the bottom of the image, to the terminal cell nucleus, at the top of image). In wild type cells the lumen tapers very gradually and evenly from proximal (bottom) to distal (top). B. In *wheezy/GckIII* mutant terminal cells, prominent transition zone (between intercellular junction and terminal cell nucleus) dilations are detected (<). Terminal cells mutant or knocked down for *tao*, *ccm3, trc* (not shown, see [20, 21]), (C) *Mo25*, or (D) *furry* show identical transition zone dilations. Scale bar = 10 microns.

DISCUSSION

The molecular mechanisms by which CCM3 acts in the vascular system remain unclear. Strong data support a role of GCKIII family kinases acting together with CCM3, but the downstream target(s) of the kinase have remained elusive. Our prior work in *Drosophila* [21] suggest that the NDR kinases (Trc in flies, STK38, STK38L in human) are likely to be the direct targets of the CCM3/GCKIII complex in endothelial cells. Studies linking the scaffolding protein Furry to Trc function implied that Furry was also likely to be required as part of the CCM3-GCKIII signaling cascade. Here we establish that at least in the fly tracheal system, the Furry/Trc complex is regulated by CCM3/GCKIII. The connection between the CCM3-GCKIII complex and a

Trc-Fry-Mob complex was suggested by *in vitro* work using human orthologs [52, 53]; however, other factors such as mTor or Hippo have also been proposed to act as upstream activators of Trc [32, 33, 40, 54]. Our data in the *Drosophila* wing demonstrate that at least in two tissues, wing and trachea, Fry/Trc are regulated by the CCM3 pathway. Further studies in additional Trc-requiring tissues, such as neurons and follicle cell epithelia, will need to be carried to determine whether the CCM3 pathway is a tissue-specific or general regulator of Fry/Trc activity. Likewise, it will be critical to extend the analysis to the vertebrate vascular system. If STK38 and STK38L are required downstream of CCM3 in endothelial cells, as expected, it will be important to identify the targets of STK38 and STK38L kinases and to determine if they are universal, differ between epithelial and neural tissues, or show even greater specificity. Given the role for CCM3-GCKIII in Trc-regulated tissue polarity in the wing, it will likewise be of interest to gain mechanistic insight into that process, and to determine whether such functions of CCM3 are related to meningiomas associated with CCM3 patients. The hypothesis that planar cell polarity (PCP) might be disturbed in meningiomas arises from the observation that a histological characteristic of meningiomas is the whorl formation of neoplastic arachnoid cells. This hypothesis has received some genetic support, as human *FAT2,* an ortholog of the *Drosophila* PCP pathway component Fat, has been implicated in meningioma [55]. Moreover, Fat protocadherens of the have been implicated in Hippo signaling [56]. In the fly, work from Horne-Badovinac and colleagues revealed that Fat2, Fry and Trc are required to polarize the follicle cell epithelia that surround the *Drosophila* oocyte. While a role for CCM3-GCKIII in the follicle cells has not yet been described, it is noteworthy that in these cells Trc was found to be distributed in a planar polarized fashion. Strikingly, it was the basolateral aspect of the cells that displayed these asymmetries in Trc distribution, raising the question of whether the mechanisms involved are related to the canonical PCP pathways, or novel.

DECLARATIONS

Authors' contributions

ASB and ASG made substantial contributions to conception and design of the study and EA-A, ASB, and ASG performed data analysis and interpretation.

Availability of data and materials

All reagents developed in the lab are available upon request.

Financial support and sponsorship

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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