

ARTICLE

Importin-9 and a TPR domain protein MpH drive periodic patterning of ciliary arrays in *Tetrahymena*

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We explored how the number of structures is determined in an intracellular organelle series. In *Tetrahymena*, the oral apparatus contains three diagonal ciliary rows: M1, M2, and M3. During development, the M rows emerge by sequential segmentation of a group of basal bodies, starting with the longest and most anterior M1 and ending with the shortest and most posterior M3. The *mpD-1* and *mpH-1* alleles increase and decrease the number of M rows, respectively. We identify MpH as a TPR protein and MpD as an importin-9. Both proteins localize to the M rows and form concentration gradients. MpH is a row elongation factor whose loss shortens all M rows and often prevents the formation of M3. MpD limits row initiation after the emergence of M2. MpD could be a part of a negative feedback loop that limits row initiation when M1 assembly is properly advanced. We conclude that the forming oral apparatus has properties of a semi-autonomous intracellular developmental field.

Introduction

The question of “how cells count?” posed by Lewis Tilney and colleagues in the title of the review about stereocilia (Tilney et al., 1992) has remained without a satisfactory answer. In ciliated protists, some cortical structures form series, and typically the number of structures in such series is reproduced with extreme accuracy across generations. Thus ciliates, and in particular the genetic model *Tetrahymena thermophila*, provide a rare opportunity to study the mechanism of precise “intracellular counting.” In *Tetrahymena* species, the oral apparatus (OA) is a “meta-organelle” that contains multiple ciliary rows whose motility directs food particles into the buccal cavity. In the population, the OA invariably has four short ciliary rows (hence the name of the species: *Tetrahymena*). Joseph Frankel and colleagues (the University of Iowa) have generated and characterized a unique collection of cortical pattern mutants in *T. thermophila*, including strains with alleles that alter the number of oral rows and, to a lesser extent, their position-dependent differentiation (reviewed in Frankel [2008]). Here, we use comparative next generation sequencing (NGS) to map two alleles: membranelle pattern D (*mpD-1*) (Frankel et al., 1984a, 1984b) and membranelle pattern H (*mpH-1*) (Frankel, 2008), which increase and decrease the number of oral rows, respectively. We find that both gene products are proteins that localize to the oral rows, where they form developmental stage-

specific concentration gradients. Microsurgery studies in the large ciliate *Stentor* dating back to the end of the 19th century, and in particular the famous studies done by Vince Tartar, have revealed that the developing OA operates with a high degree of autonomy. For example, a new OA can regenerate when most of the cell cortex (including the preexisting OA) is removed (Tartar, 1956b, 1961) or when a narrow region of the cell cortex containing an early stage oral primordium (OP) is transplanted into a “wrong” place in another cell (Tartar, 1956a). Here, we describe two proteins that localize to the OA and regulate the size of the oral field. MpH expands the field laterally by promoting row elongation. MpD/importin-9 inhibits row initiation and reduces the anterior-posterior (AP) length of the field. Our observations support a model that in a ciliate, oral morphogenesis is executed within a semiautonomous intracellular developmental field.

Results

Oral development in the wild-type *Tetrahymena*

In the *Tetrahymena* species, the OA (Fig. 1 A) contains four compound ciliary rows: three diagonal so-called “membranelles” (M1, M2, and M3) and the orthogonal so-called “undulating membrane” (UM) positioned along the right¹ margin of the M

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¹“Left and right” are based on the cell’s perspective.

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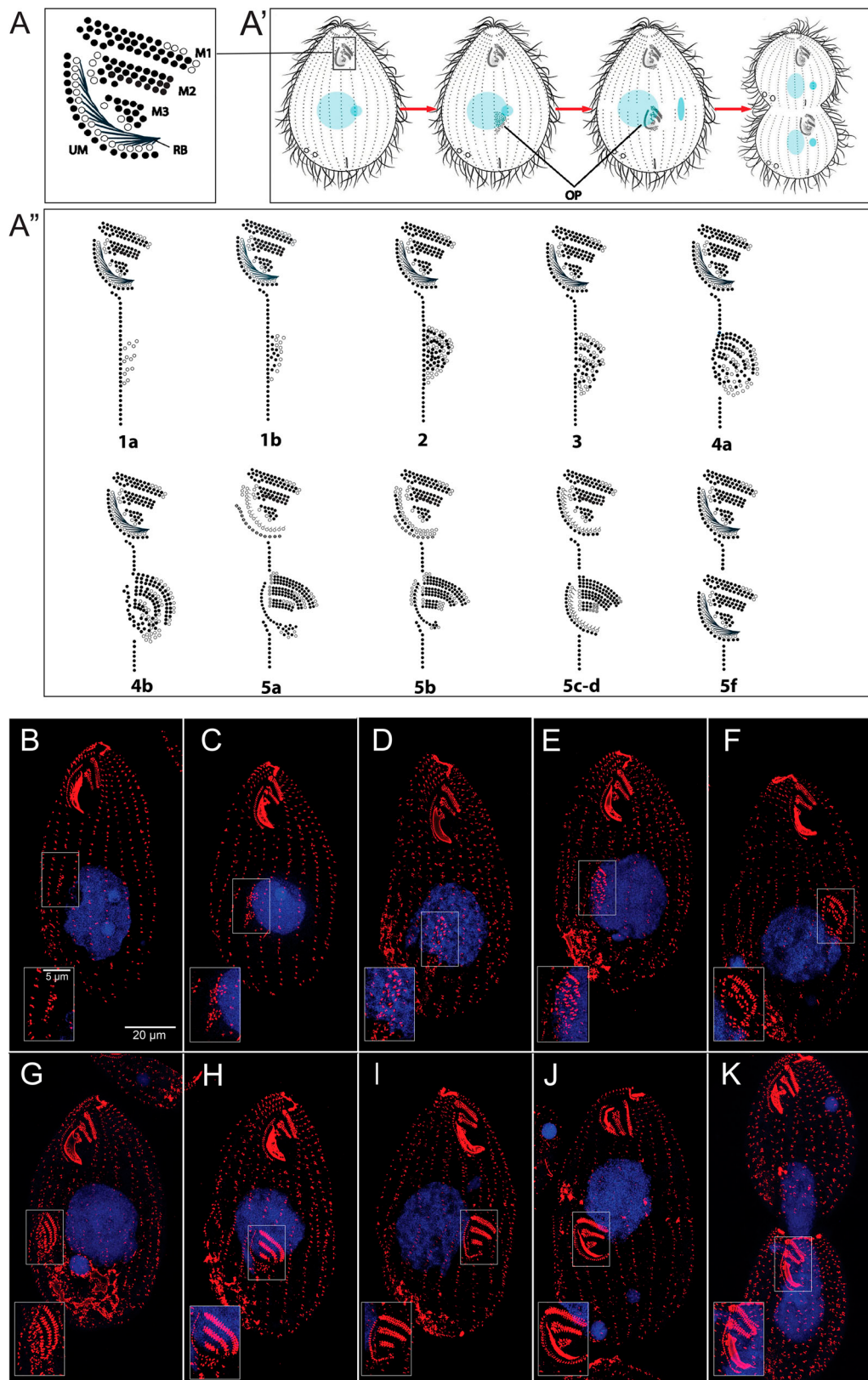


Figure 1. Oral development in WT *Tetrahymena*. (A) The structure of the OA. (A') The course of cell division (tandem duplication). The following stages are shown from left to right: interphase, cell division stages 3 and 5, and cytokinesis. (A'') Stages of divisional oral development (based on Lansing et al. [1985]; Nelsen [1981]). See Table 1 for details. In panels A and A' the subtypes of BBs are displayed as follows: filled circles; ciliated BBs, open circles; unciliated BBs, crossed circles; BBs destined for resorption. (B–K) SR-SIM images of WT *Tetrahymena* cells stained with 20H5 anti-centrin (red) and DAPI (blue). Insets show the OP region at a higher magnification. (B) Early stage 1a. (C) Late stage 1a. (D) Stage 1b. (E) A transition between stages 2 and 3. (F) A transition between stages 3 and 4a. (G) Stage 4b. (H) A transition between stages 4b and 5a. (I) Stage 5a. (J) Early stage 5f. (K) Late stage 5f. RB, ribbed wall of microtubules; OP, oral primordium.

row ends (reviewed in Frankel [1989]; Wloga and Frankel [2012]). This study is focused on the mechanism that determines the number of M rows. There is gradation in the M row length ($M1 > M2 > M3$) (Fig. 1 A). M1 and M2 are structurally similar as their cores are formed mostly by columns of basal body (BB) triplets, while the most posterior M3 is non-columnar (Fig. 1 A). While the majority of BBs in the M rows are ciliated, a few BB at specific positions (Fig. 1 A, open circles) lack cilia (Bakowska et al., 1982a, 1982b; Lansing et al., 1985; Williams and Bakowska, 1982). The UM is composed of two rows of BBs, but only the outer row is ciliated, while the inner row is connected to a set of microtubules that project toward the bottom of the buccal cavity, forming the so-called “ribbed wall” (RB in Fig. 1 A) (Lansing et al., 1985; Nelsen, 1981).

Like most ciliates, *Tetrahymena* cells divide by a type of binary fission called “tandem duplication” (Fig. 1 A’), in the course of which the cell is remodeled into two daughters having the same AP polarity (reviewed in Cole and Gaertig [2022]; Soares et al. [2019]). The anterior hemi-cell inherits the old OA, while a new OA (that we will refer to as the oral primordium, OP) assembles in the posterior hemi-cell. Fig. 1 A” shows the stages of divisional oral development (after Lansing et al. [1985]; Nelsen [1981]). We found most of these stages in the wild-type (WT) *T. thermophila* using the anti-centrin antibody 20H5 (Salisbury et al., 1988) as the BB marker and super-resolution structural illumination microscopy (SR-SIM) (Fig. 1, B–K).

We will briefly review the course of oral development with the focus on the M rows (see Table 1 for a more complete description). At the onset of cell division, the OP starts to form as a group of newly assembled and unciliated BBs that appear on the left side of the right postoral somatic ciliary row at a roughly equatorial position along the cell’s A/P axis (Fig. 1 A”-1a and Fig. 1 B). The BBs of the OP field proliferate and populate the space between the two postoral rows (Fig. 1 A”-1b; and Fig. 1, C and D). The BBs undergo maturation and become ciliated. New BBs appear at anterior positions to the old ciliated BBs, producing BB pairs (Fig. 1 A”-2 and Fig. 1 E). A side-by-side alignment of the BB pairs generates M rows, with the most anterior M1 emerging first, followed by M2 and M3 (Fig. 1 A”-3, 4a; and Fig. 1, E and F). The third BB subrow forms in each M row by nucleation of a new BB at the most anterior position, which produces BB triplet columns. The formation and ciliation of the third subrow progresses from right to left and is most advanced in M1 and least advanced in M3 (Fig. 1 A”-4b and Fig. 1 G). During stages 5a–5f, the M rows that so far differed mostly in their length undergo differentiation. The row-specific steps include the formation of a limited fourth subrow in M1 (Fig. 1 A”-5a), resorption of specific BBs at either the right or left end of each M row, and resorption of some cilia resulting in unciliated BB (Fig. 1 A”-5a–5d; and Fig. 1, H and I). Finally, during the “sculpturing” phase (Fig. 1 A”-5f; and Fig. 1, J and K), several previously columnar BBs (located at the rows right ends) move apart. The direction and extent of the BB movements are row specific, and consequently, each row acquires a unique pattern of BBs. The shortest M3 row undergoes the most extensive sculpturing and no longer has columnar BBs. The parental (old) OA remains morphostatic until stage 5a,

Table 1. Stages of oral development in *Tetrahymena* (after Lansing et al. [1985]; Nelsen [1981])

Stage	nOA	Old OA
1a	Strings of BBs form off of several somatic BBs in the right postoral row	
1b	New BBs populate the space between the two postoral rows and have random orientations in regard the cell’s AP axis	
2	BBs undergo duplication and become pairs composed of an older ciliated posterior BB and a younger unciliated anterior BB	
3	The BB pairs associate side-by-side to form M rows. M1 and M2 become visible	
4a	The most posterior M3 emerges. In all M rows, the anterior BBs undergo ciliation, starting from the row’s right end and progressing toward the left UM merges as a file of single BBs that move away from the rest of the oral field toward the right margin of the OP. At the posterior end of the OP, there is a large group of mostly unciliated, newly formed BBs. This area is already present in stage 2 and is likely the site of continuing proliferation of BBs, which are subsequently added to the forming rows	
4b	A third most anterior subrow of BBs starts to form at each M row’s right end and progresses to the left. The formation of the third subrow is most advanced in M1 and least advanced in M3 The UM fully separates from the rest of the oral field except for the most posterior area containing young unciliated BBs to which all 4 row ends connect to	
5a	The assembly of the third most anterior subrow has completed in all M rows. The ciliation of the third subrow continues right to left. The M rows condense by reduction of spaces between the BB columns and became less curved. In M1, two new BBs form a limited fourth subrow. A single triplet of BBs is resorbed at the right end of each M row The UM is now composed of a single row, while the posterior field of new BBs is small and located near the posterior end of the UM	Initiation of limited remodeling of the old OA. The ribbed wall microtubules disassemble. The outer UM row moves slightly to the cell’s right side, and a new outer row starts to form at the anterior end of the UM

Table 1. **Stages of oral development in *Tetrahymena* (after Lansing et al. [1985]; Nelsen [1981]) (Continued)**

Stage	nOA	Old OA
5b	In the M rows, several BBs that were previously ciliated, loose cilia. Especially prominent deciliation occurs at the left end of M3, where it affects two terminal BB triplets The outer row starts to form in the UM	The old outer row of UM starts to be resorbed. The new UM outer row is completed, and its BBs undergo ciliation
5c-d	Resorption of specific BB on the left side of M rows, including the two leftmost BBs in the most anterior subrow of M1 and a total of 7 BBs at the left end of M3 The new UM has two full-length rows, and the outer row is fully ciliated. In contrast, cilia in the inner UM row resorb. The ribbed wall microtubules start to grow out of the inner UM row	In the old UM, the degradation of the outer row is complete, and the new outer row is completely ciliated. The ribbed wall microtubules grow out of the inner UM row
5f	The M rows undergo sculpturing of their right ends that involves displacements of specific BBs resulting in the dissolution of their triplet columns. The sculpturing of M3 is most extensive, and the BB columns are no longer apparent The ribbed wall is fully developed	The ribbed wall assembly is completed

when its UM undergoes limited remodeling (Fig. 1 A'-5a-5f and Table 1).

***mph-1*, an allele that reduces the number of M rows, is a mutation in a TPR domain protein**

About half of *Tetrahymena* mutant cells homozygous for *mph-1* (Frankel, 2008) assemble an OA with 2 instead of normal 3 M rows ($46.8 \pm 6.2\%$ $N = 3$ experiments, 100–130 cells scored per experiment), while no such cells were found in the WT ($N = 3$ experiments, 100 cells scored per experiment). Furthermore, the mutant M rows are excessively short (Fig. 2, B–D compare with Fig. 2, A and P). It appears that in the mutant OAs with 2 M rows, the missing row is M3. For example, in Fig. 2, C and D, the 2 M rows present are positioned close to the anterior margin of the OA, and no row is present along the ribbed wall where M3 is located in the WT (see Fig. 1 A). The row-specific sculpturing patterns were frequently abnormal (Fig. 2, B and C) or the sculpturing features were not apparent (Fig. 2 D). During divisional oral development, between stages 3–5 days, in most OPs three rows could be discerned (Fig. 2. I–K). However, in a subset of cells in stage 5f, in place of an M3 row there was a group of dispersed BBs that failed to form a row, suggesting a late row formation defect that affects M3 (Fig. 2 L compare with Fig. 1 J). In addition, *mph-1* mutant cells frequently undergo oral replacement (OR), an alternative mode of oral development that occurs without cell division, which in the WT is triggered by

starvation (Frankel, 1969; Kaczanowska et al., 2008). During OR, an OP forms near the posterior end of the old OA. As the OP develops, the old OA undergoes resorption. In the WT, the stages of oral development during OR are similar to those occurring during divisional oral development (Frankel, 1969; Kaczanowska et al., 2008). However, the *mph-1* mutant homozygotes undergo OR in the nutrient medium, possibly because this pathway is triggered by the lower rate of feeding. On average, $16.7\% \pm 4.9\%$ of *mph-1* homozygotes were undergoing OR ($N = 3$ experiments, 100–130 cells scored per experiment) while such cells were not found in the WT population ($N = 3$ experiments, 100 cells scored per experiment). Fig. 2, M–O shows images of *mph-1* mutants undergoing OR. In the cell shown in Fig. 2 O, the OP is between stages 3; it is excessively small, and only 2 M rows can be discerned. Overall, the *mph-1* phenotype is a combination of M row shortening and a frequent failure to assemble the last forming, most posterior M3, and these defects occur either during cell division or OR.

To map *mph-1*, we used a comparative NGS approach, the allelic composition contrast analysis (ACCA) (Jiang et al., 2017), to search for genomic variants that co-segregate with the mutant phenotype among meiotic segregants. A single linkage peak was detected on the micronuclear chromosome 1 (chr1) at ~7 Mb (Fig. 3 A). Within this region, variant chr1:7220586 T/C, was altered in 100% of the mutant reads ($N = 33$) and was 83% reference among the WT reads ($N = 42$). In the macronuclear genome, this variant is located in the *TTHERM_00564170* gene, which encodes a protein with an N-terminal IDR domain followed by 15 tetratricopeptide repeat (TPR) motifs (Fig. 3 B). AlphaFold2 (Jumper et al., 2021; Mirdita et al., 2022; Varadi et al., 2022) predicted that the TPR domain forms an α -solenoid (Fig. 3, C and D). A BLASTp search of the aa sequence of *TTHERM_00564170* against the human proteome revealed a moderate homology with TTC6, limited to the TPR domain. Within the predicted coding region of the macronuclear *TTHERM_00564170* (confirmed by the RNA-seq data at the TetraFGD database [Xiong et al., 2011]), chr1:7220586 T/C results in the A5354G substitution, two bases upstream of the 3' splice junction of intron 8, which is expected to create a premature splice site and change the translational reading frame, resulting in a premature stop codon and replacement of 179 C-terminal aas by 16 novel aas (Fig. S1 A). Sequencing of a reverse-transcribed fragment of *TTHERM_00564170* mRNA isolated from the mutant strain (IA483) confirmed the expected change in the 3' splice site position (Fig. S1 B). While the affected C-terminal region is outside of both the IDR and TPR domains, its aa sequence is moderately conserved in *TTHERM_00564170* orthologs of other ciliate species, including *Stentor coeruleus* and *Paramecium tetraurelia* (Fig. S2). We tested whether *TTHERM_00564170* is the sought MPH gene by replacing the variant base with a WT base in the *mph-1* homozygotes. To this end, we used homologous DNA recombination to target *TTHERM_00564170* in the *mph-1* mutant strain IA483 with a DNA fragment that contained a portion of the WT *TTHERM_00564170* coding region that covers the variant site, followed by a sequence of GFP-mNeonGreen, *neo5* marker and a fragment of the 3' UTR. Thus, the targeting fragment was intended to replace the variant base and add the

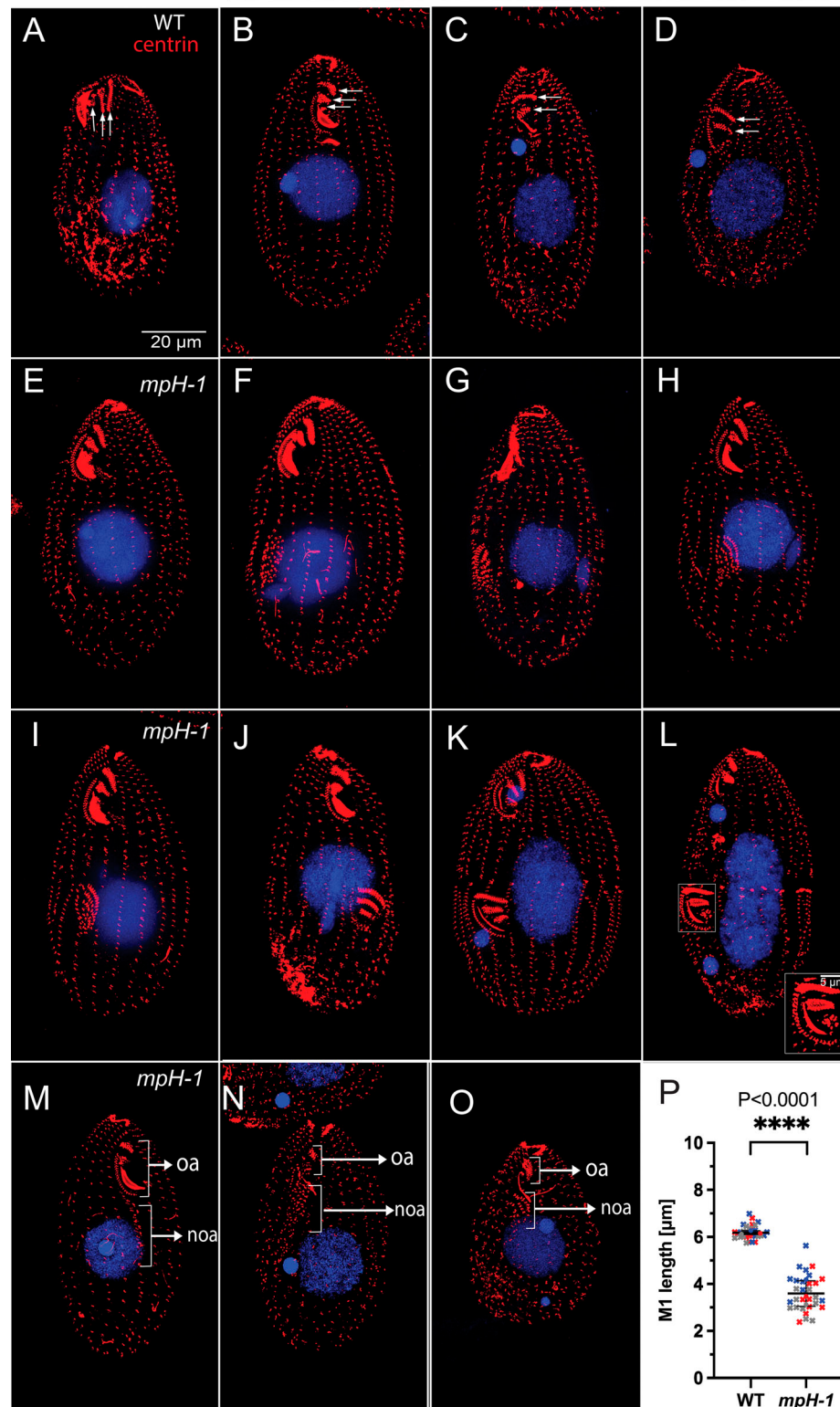


Figure 2. ***mpH-1* confers shortening of M rows and loss of M3.** (A–O) SR-SIM images of a WT (A) and *mpH-1* homozygote cells (B–O) grown at 30°C and immunostained with 20H5 anti-centrin (red) and DAPI (blue). In panels A–D, white arrows mark the M rows. (B–D) *mpH-1* mutant cells in interphase. The cell in B has a highly reduced M3. The cells in C and D lack M3. (E–L) *mpH-1* cells during divisional oral development. (E) Stage 1a. (F) Stage 1b. (G) Stage 2. (H) Stage 3. (I) Stage 4a. (J) Stage 5a. (K) Early stage 5f. (L) Late stage 5f. (M–O) *mpH-1* cells that undergo OR. (M) Stage 1b. (N) Stage 2. (O) Stage 4a. (P) Quantification of the length of M1 row. Mean $\pm$ SD ($3.6 \pm 0.8 \mu\text{m}$ in the *mpH-1* homozygotes and $6.19 \pm 0.3 \mu\text{m}$ in the WT). $N = 3$ experiments (10 measurements per experiment). $P < 0.0001$ in a two-way ANOVA test with Geisser–Greenhouse correction for variability. oa, old oral apparatus in various stages of resorption during oral replacement; noa, new oral apparatus in cells undergoing OR.

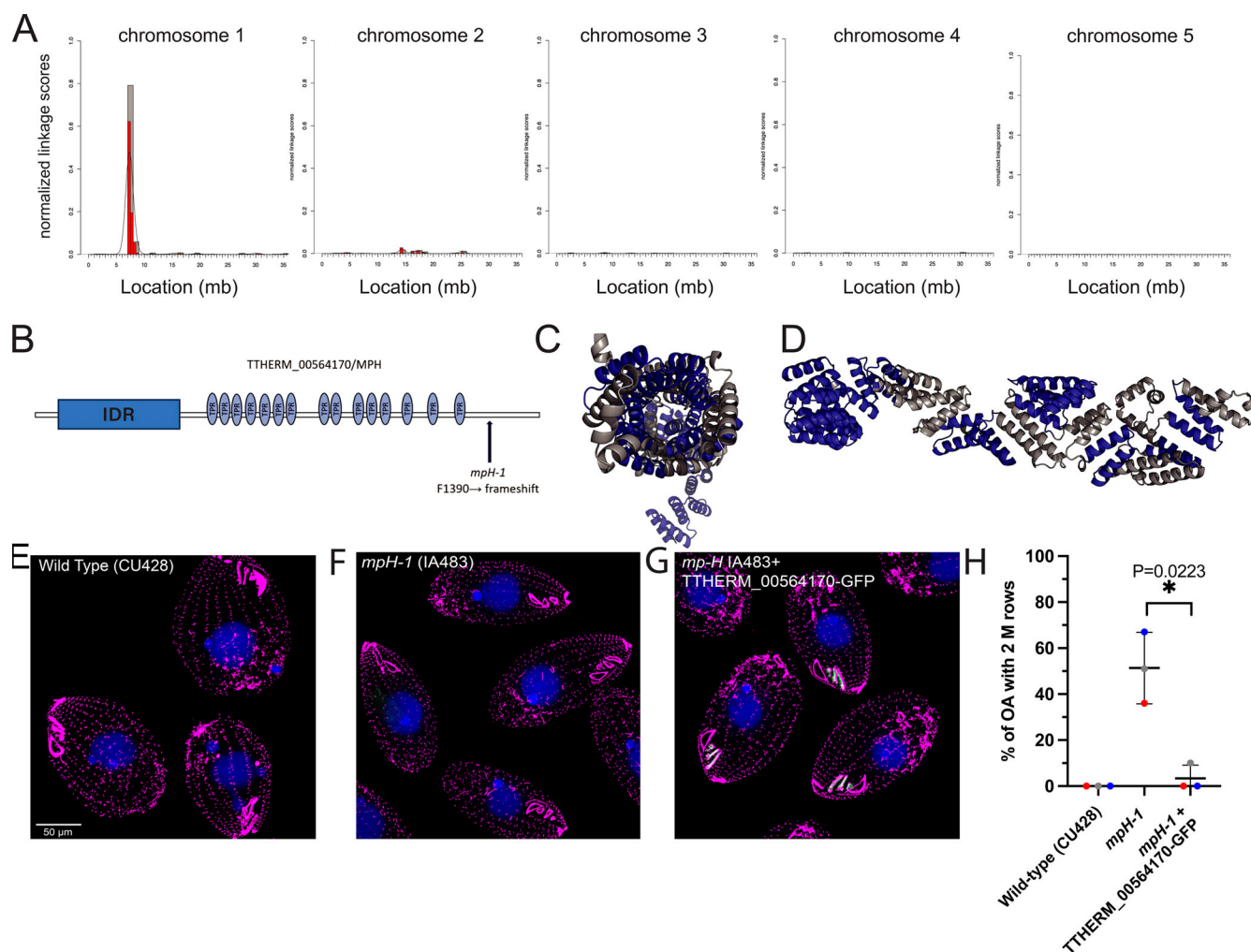


Figure 3. *mph-1* is conferred by a mutation in *THERM_00564170*. (A) NGS-based mapping of variants co-segregating with *mph-1* in the micronuclear genome using the ACCA method. *mph-1* is linked to a region on chr1 around 7 Mb. (B) The domain organization of THERM_00564170 protein. (C and D) An AlphaFold2 model of the TPR domain of THERM_00564170 viewed from the protein's C terminus (C) and from the side (D). The TPR segments are shown in blue. (E–H) Replacement of the A5354 variant base by the reference G base in the *mph-1* homozygotes rescues the mutant phenotype. (E–G) SR-SIM images of cells, grown at 30°C and stained with 20H5 anti-centrin (magenta) and DAPI (blue). GFP-mNeonGreen fluorescence was imaged directly (green). (E) WT; F, *mph-1* homozygotes; G, *mph-1* homozygotes with an introduced Mph-GFP-mNeonGreen fragment that replaced the A5354 variant base with the WT G and added Mph-GFP-mNeonGreen sequence to the 3' end of the coding region of THERM_00564170 (see Fig. S1 C). (H) Quantification of the percentage of OA with 2 M rows in strains with the following macronuclear genotypes: WT (strain CU428), *mph-1* (strain IA483), and *mph-1* with A5354 variant base replaced by the reference G and GFP-mNeonGreen at the end of the coding region of THERM_00564170. Mean ± SD. N = 3 experiments indicated by symbol colors (15–124 cells scored per experiment, a total of 112–226 cells scored per genotype). P = 0.0223 in an unpaired *t* test with Welch's correction.

tag. We analyzed 21 paromomycin (pm)-resistant transformant clones, and among them, 7 were phenotypically rescued and 13 retained the mutant phenotype. Out of the 7 rescued clones, 6 showed a strong GFP-mNeonGreen fluorescence in M rows (Fig. 3 G compare with Fig. 3 F and Fig. S1 E). In contrast, all 13 non-rescued transformants lacked fluorescence above the background (Fig. S1 D). Sequencing of the THERM_00564170-coding region in the non-rescued transformants (N = 2) revealed that the GFP-mNeonGreen-neo5 sequence was inserted at the end of the coding region carrying an altered base. In contrast, in the phenotypically rescued transformants that showed the M row fluorescence, the GFP-mNeonGreen-neo5 sequence was inserted downstream of the reference base (N = 2) (Fig. S1 C). The presence of either the variant or reference base upstream of

the GFP-mNeonGreen-neo5 sequence can be explained by different crossover positions during gene replacement events. Sequencing of the single phenotypically rescued transformant-lacking GFP-mNeonGreen fluorescence revealed two alleles, one with the reference base but lacking the linked GFP-mNeonGreen-neo5 sequence and another allele with the altered base upstream of the GFP-mNeonGreen-neo5 sequence. Multiple recombination events in the same cell are possible because in the macronucleus, genes are present in ~90 copies (Zhou et al., 2022). Overall, replacement of the altered base by the reference base correlates with phenotypic rescue (Fig. 3 H). Also, when GFP-mNeonGreen-coding sequence was present downstream of the variant base, the fusion protein was not expressed (Fig. S1 D), consistent with the predicted change in the reading

frame. Altogether, these results led us to conclude that *THERM_00564170* is *MPH*.

MpH forms concentration gradients within the developing OP that correlate with the BB age and M row maturity

We analyzed the pattern of MpH-GFP-mNeonGreen expressed in the native locus. In the old OA, MpH-GFP-mNeonGreen was strongly present in all 3 M rows, and remarkably, not detectable in the UM (Fig. 4 A). In addition, in most cells, dots of MpH-GFP-mNeonGreen (usually two) were present at the apical cell end (arrowheads in Fig. 4, B–D), anterior to the apical crown (ac), an incomplete ring of BB pairs located at the anterior ends of a subset of somatic ciliary rows (Jerka-Dziadosz et al., 2001; Wloga and Frankel, 2012). During divisional oral development, MpH-GFP-mNeonGreen appeared in the OP in stage 1a as several small foci (Fig. 4 B). In stage 1b, MpH-GFP-mNeonGreen dots accumulated preferentially on the left side of the OP and most dots colocalized with the BBs (Fig. 4 C). In stage 3, the M rows form by side-by-side alignment of the BB pairs; MpH-GFP-mNeonGreen presented as rings paired with centrin dots (Fig. 4 D). Based on the known orientation of BBs in the oral field at stage 3 (Bakowska et al., 1982b; Lansing et al., 1985), the MpH-GFP-mNeonGreen rings colocalized with the posterior (older) while the centrin dots marked the anterior (younger) member of the BB pair, respectively. In stage 4b, MpH-GFP-mNeonGreen marked all 3 M rows, but there was a gradient in intensity across the oral field, with the strongest signal in M1 and the weakest signal in M3 (Fig. 4 E). The simplest interpretation is that the gradient corresponds to the degree of maturity of BBs forming the M rows. At stage 4b, M1 and M2 rows gain a third most anterior BB subrow (more complete in M1) (Lansing et al., 1985). There was a gradient of intensity of MpH-GFP-mNeonGreen across the subrows, best visible in M2: MpH-GFP-mNeonGreen was strongest in the most posterior subrow, weaker but still present in the middle subrow, and not detectable in the anterior (youngest) subrow (inset in Fig. 4 E). Again, the intra-row gradient fits with the age of the BBs. In the late stages 5(c–f), the M rows undergo sculpturing at their right ends that is row specific (Frankel et al., 1984a, 1984b). During the sculpturing phase, the pattern of MpH-GFP-mNeonGreen became more variable. Initially, the inter-row gradient was apparent (Fig. 4 F), but later on the gradient direction was reversed, with the highest point at M3 (Fig. 4, G and H). To conclude, MpH colocalizes with the M rows starting from the earliest stage of OP development, and at multiple stages the levels of MpH correlate with the age of BBs and consequently reflect the row maturity.

Overexpression of MpH promotes row assembly

The M row-specific localization within the OA and the observed row shortening in the *mpH-1* mutant cells suggested that MpH promotes row assembly and specifically row elongation. To test this model, we overproduced GFP-MpH using the cadmium-inducible *MTT1* promoter (Shang et al., 2002). 6 h after addition of cadmium chloride to the medium, GFP-MpH strongly accumulated within the mature OA and OP in dividing cells but remained restricted to the M rows and spaces between them (Fig. 5, A–B"). Two effects of overexpression were observed.

First, frequently the OPs contained fragmented M rows (Fig. 5 C). For example, the dividing cell shown in Fig. 5, A–A" has a nearly normal mature OA and an OP with ~10–12 M rows. In that OP, the UM is not apparent, and the ribbed wall (rw in Fig. 5 A') is also fragmented. While the M row fragmentation effect could be explained by elevated initiation activity of M rows, an alternative explanation is that excessive MpH causes row assembly defects. The second phenotype was a modest but significantly increased M row length (Fig. 5 D). Overall, we conclude that overexpression of MpH stimulates M row assembly.

mpD-1 confers the formation of extra M rows during oral development

mpD-1 is a conditional recessive allele whose expression at elevated temperature (39°C) increases the number of M rows from three to four and sometimes even five (Frankel et al., 1984a, 1984b). Despite the increased row number, the row organization (including the length and sculpturing pattern) tends to agree with the row's AP position (Frankel, 2008), suggesting that the mutation affects the row number determination and not the subsequent row differentiation. All examined WT cells and most *mpD-1* homozygotes have 3 M rows at the standard 30°C temperature (Fig. 6, A and B). After 6 h of multiplication at 39°C, in the *mpD-1* homozygotes, 73% of OAs had 4 M rows ($72.7 \pm 3.5\%$, $N = 3$ experiments, 100 cells scored per experiment), and no such OAs were present in the WT (Fig. 6, C, D, and J). As was the case of *mpH-1* mutants, *mpD-1* mutant cells grown at 39°C develop a nOA either during cell division or OR ($5.1 \pm 1.7\%$ of growing population, $n = 3$ experiments, 100 cells scored per experiment). The extra rows appear in course of both divisional development and OR (see below and Fig. S3).

To determine how the extra M rows form, we compared the course of oral development between the dividing WT and the *mpD-1* homozygotes at 39°C. The early stages (1–3) were not visibly affected (Fig. 7 A–C'). In the mutants, 4 M rows could be discerned at stage 4a, which is the time when M3 emerges in the WT (Fig. 7, D and D'). Because the extra row, M4, tended to be very short at stage 4a, we examined the row pattern in an *mpD-1* strain that also expresses MpH-GFP-mNeonGreen, our now established M row marker. In the mature OAs with 4 M rows, MpH-GFP-mNeonGreen was indeed present in all of them (Fig. S4, A–A"). During divisional oral development before the row emergence (stage 2), MpH-GFP-mNeonGreen was properly enriched on the left side of the primordium, where M1 starts to form (Fig. S4, B–B"). In stage 4a, four MpH-GFP-mNeonGreen-positive rows were visible (Fig. S4, C–D"). We showed above that MpH accumulates in the developing M rows in a manner reflecting the row maturity. In the WT, in stage 4b, the last emerging row M3 has the lowest level of MpH (Fig. 4 E). In agreement, in the *mpD-1* mutant cells with 4 M rows, the shortest most posterior row had the weakest signal of MpH-GFP-mNeonGreen (Fig. S4, C" and D"). The simplest interpretation for the origin of the extra row is that an additional most posterior row forms at the time or shortly after the formation of M3. Thus, the WT cell has an overcapacity for row initiation that is normally suppressed by MpD.

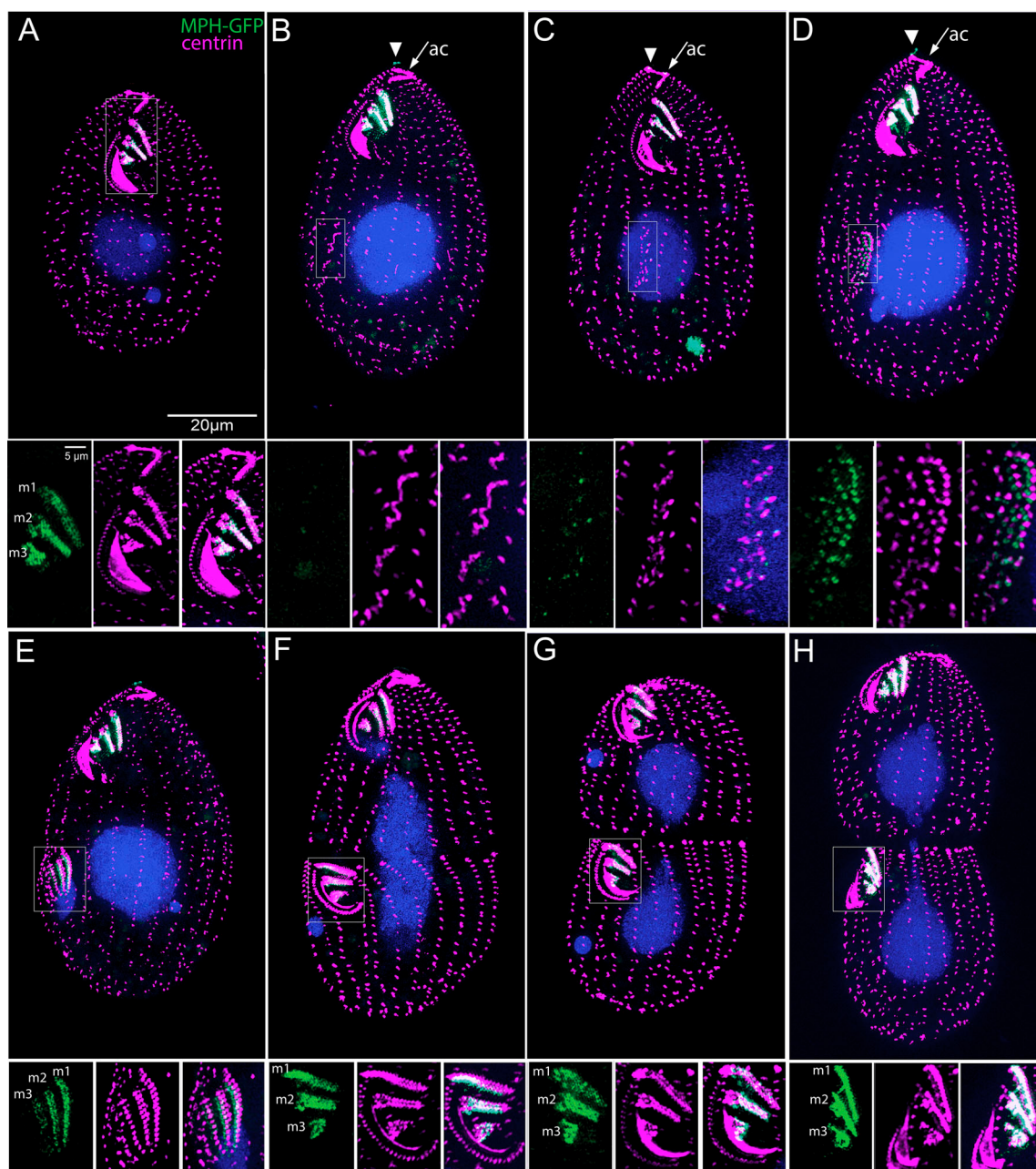


Figure 4. MpH localizes to M rows and forms gradients that reflect the time of BB maturation. (A–H) SR-SIM images of cells expressing MpH-GFP-mNeonGreen (in the native locus, green), stained with the anti-centrin (magenta) and DAPI (blue). The MpH-GFP-mNeonGreen fluorescence is detected directly (green). Insets show either the mature OA (A) or the OP region (B–H) at a higher magnification. **(A)** Interphase. Note the presence of MpH in the 3 M rows, not in the UM. **(B)** Stage 1a. **(C)** Early stage 1b. MpH-GFP-mNeonGreen foci are enriched near BBs on the left side of the OP. **(D)** Stage 2. BB pairs align into M rows, with M1 partly formed. MpH-GFP-mNeonGreen presents as rings corresponding to the position of the older (posterior) BB in the pair. The centrin foci are at positions consistent with the anterior (younger) BB of the pair. Note the gradient of MpH-GFP-mNeonGreen intensity between the M rows, with M1 at the gradient's high point. **(E)** Stage 4b. Within the M rows, MpH-GFP-mNeonGreen is abundant in the first (oldest and most posterior) and second (intermediate) subrows but weakly present or not detectable in the third (youngest) subrow. The M1-M2-M3 gradient across the oral field is also apparent (see inset). **(F)** Early stage 5f. **(G)** Intermediate stage 5f. MpH-GFP-mNeonGreen present in all three subrows. Note a reversed gradient of intensity M3>M2>M1 also seen in the old OA in interphase (see panel A). **(H)** Late stage 5f. m1, m2, and m3; right ends of M rows marked in insets; ac, apical crown. The arrowheads in panels B–D mark MpH-GFP-mNeonGreen foci at the apical cell.

MPD encodes an ortholog of importin-9

We use ACCA to map *mpd-1*, which revealed a weak linkage signal on the micronuclear chromosome 3 (chr3) around 20 Mb (Fig. 6 E). When the variants were mapped to the macronuclear reference genome, two linkage signals were present, on chr_105

and chr_108, that are colinear with portions of the 20–22-Mb region on the micronuclear chr3 (Eisen et al., 2006; Hamilton et al., 2016; Wang et al., 2021). Importantly, previously, crosses between *mpd-1* homozygotes and nullisomic strains (Bruns et al., 1983; Bruns and Brussard, 1981) mapped *mpd-1* to the right arm

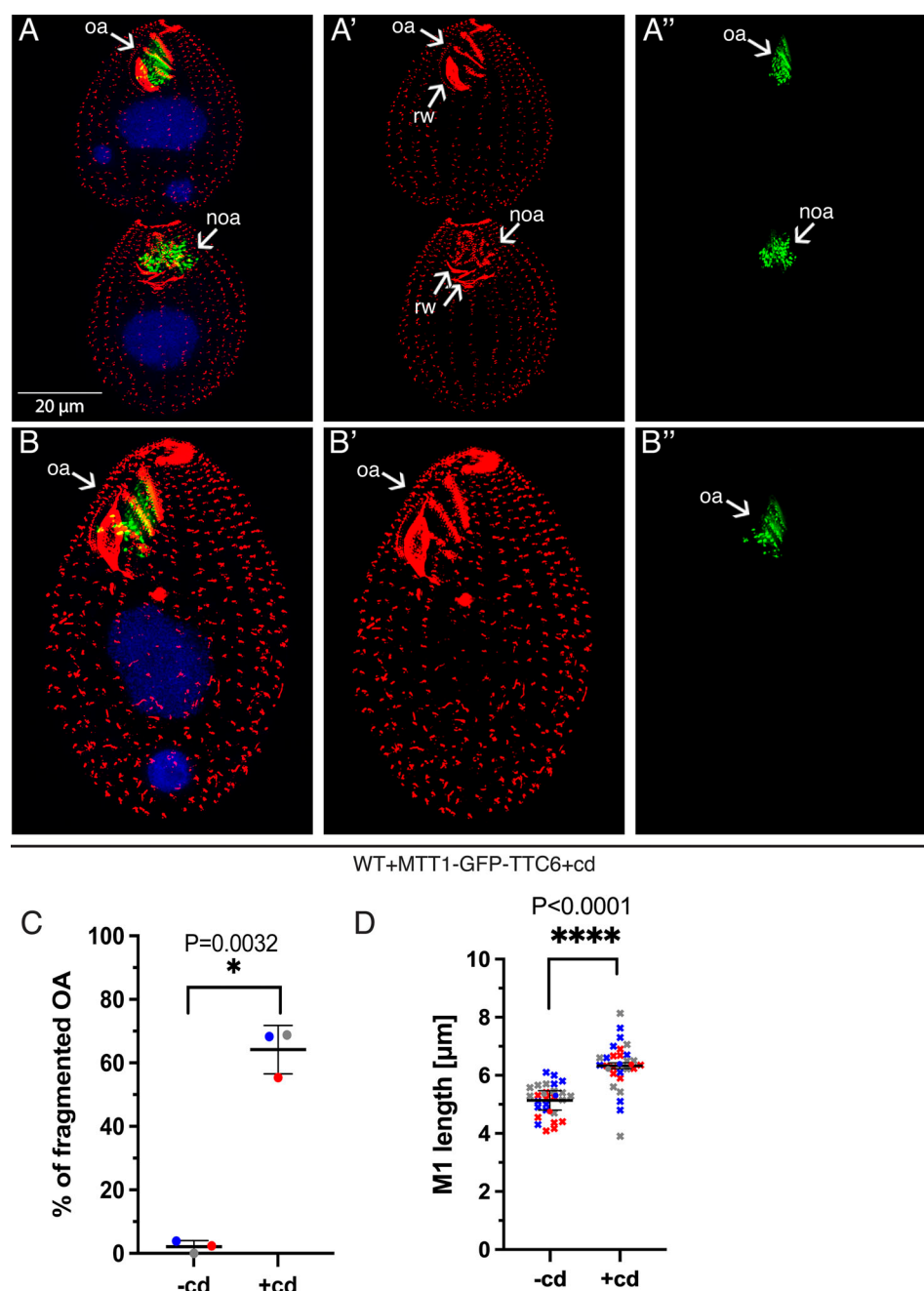


Figure 5. Overexpression of GFP-MpH promotes M fragmentation and elongation. The CU428 WT strain was modified by replacing the native promoter of *MPH* with the cadmium-inducible promoter *MTT1* to drive expression of GFP-MpH. **(A–B'')** Two sets of SR-SIM images of cells overproducing GFP-MpH after exposure to cadmium chloride 2.5 μg/ml for 6 h. The cells were labeled with 20H5 anti-centrin (red) and DAPI (blue); GFP-MpH (green) was detected directly. Note the fragmented OP in the dividing cell in panel A–A''. **(C)** Quantifications of the percentage of cells with OAs having fragmented M rows. Mean ± SD. *N* = 3 independent experiments indicated by symbol colors (100 cells scored per experiment). *P* = 0.0032 in an unpaired *t* test with Welch's correction. **(D)** Quantification of the length of M1 in cells carrying the *MTT1*-GFP-MpH transgene that were grown without (–cd) or with (+cd) 2.5 μg/ml cadmium chloride for 6 h to induce overexpression. Mean ± SD. *N* = 3 experiments. The individual measurements made in each experiment are marked by different symbol colors (9 cells measured per experiment, a total of 27 per condition). *P* < 0.0001 in a two-way ANOVA test with Geisser–Greenhouse correction for variability. oa, parental oral apparatus; noa, new oral apparatus or OP; rw, ribbed wall.

of chr3 (Frankel, 2008), which is consistent with the linkage signal position (Fig. 6 E). Within the mapped micronuclear region, chr3:20104818 G>A variant was fully homozygous for the altered base in the mutant progeny F2 pool (*n* = 132 reads), while in the WT F2 pool 88% of reads were reference (*n* = 86 reads).

This variant is predicted to cause the G939R substitution in THERM_0077076/IMP9, a protein with an importin domain. Next, we sequenced the *IMP9* gene in the strain homozygous for *mpD-2* allele (Frankel, 2008), which revealed a variant that causes the D1148N substitution. We directly tested whether *IMP9*

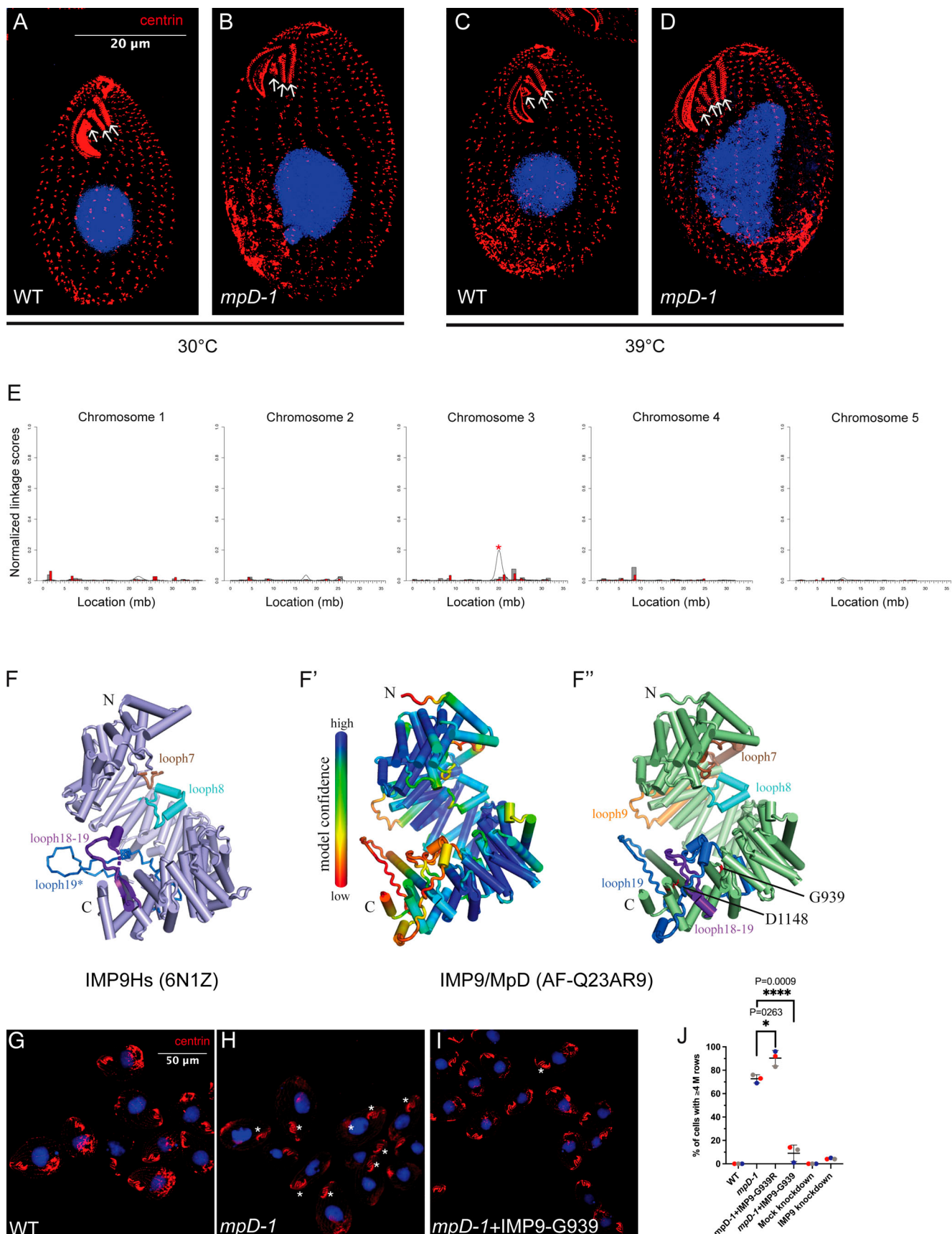


Figure 6. ***mpD* alleles are conferred by mutations in *IMP9*.** (A–D) *mpD-1* increases the M row number. SR-SIM images of cells stained with the anti-centrin (red) and DAPI (blue), WT or *mpD-1* homozygotes were grown at either standard temperature 30°C or 39°C for 6 h. White arrows mark the ends of M rows. (E)

Mapping of *mpd-1* in the micronuclear genome using ACCA. Note a small linkage peak on chr3 at ~20 Mb (red star). **(F–F’)** A comparison of human IMP9 (IMP9Hs) structure with the AlphaFold model of *Tetrahymena* IMP9/MpD. **(F)** The experimental (crystal) structure of H2A-H2B-bound IMP9Hs (PDB: 6N1Z) shown in cartoon representation, with the bound histone H2A-H2B cargo removed to focus on the structure of importin-9 (light purple). IMP9Hs is a superhelical/solenoid protein made up of 20 HEAT repeats, each comprising a pair of alpha helices. Most of the IMP9Hs alpha helices are connected by short loops, but four loops are long and colored differently: h7loop (brown), h8loop (aquamarine), h18–19loop (dark purple), and h19loop (blue). An asterisk labels h19loop because it was not resolved in the crystal structure. Instead, we superposed the h19loop from the AlphaFold model of IMP9Hs (AF-Q96P70) onto the crystal structure (PDB: 6N1Z). **(F’ and F’')** Cartoon representations of the predicted model of *Tetrahymena* IMP9/MpD by AlphaFold (AF-Q23AR9). The model in F’ is colored by the confidence score for each residue of the predicted AlphaFold model. F’’ shows the same AlphaFold model colored according to the HEAT repeat helices (green) and the long loops (colored like the homologous IMP9Hs loops). The predicted h9loop of *Tetrahymena* IMP9/MpD is longer than in human IMP9 and colored light orange. In general, the AlphaFold model of *Tetrahymena* IMP9/MpD is very similar to the structure of human IMP9 shown in F. Residues G939 and D1148, which are mutated in *mpd-1* (G939R) and *mpd-2* (D1148N), are marked with arrows. **(G–J)** Genetic tests show that the *IMP9* gene is *MPD*. **(G–I)** Confocal images of *Tetrahymena* cells with the indicated genotypes (grown at 39°C) and labeled by the anti-centrin antibody (red) and DAPI (blue). Asterisks mark OAs with 4 M rows characteristic of *mpd-1*. Cells shown in I are *mpd-1* mutant homozygotes in which the variant codon R939 was replaced by the reference G codon via homologous DNA recombination (Fig. S5J, lane 6). Note a reduction in the presence of OAs with 4 M rows. **(J)** The graph quantifies the extra M row phenotype in strains with the indicated genotypes: WT; *mpd-1*, mutant homozygote (strain IA305); and *mpd-1*+IMP9-G939R, an *mpd-1* homozygous strain carrying the *mpd-1*-linked variant (G939R codon substitution) with GFP sequence integrated at the end of the coding region of *IMP9*. Note that the frequency of OAs with 4 M configuration is higher than in the original *mpd-1* homozygote strain, possibly because addition of GFP further decreased the functionality of G939R MpD; *mpd-1*+IMP9-G939; an *mpd-1* homozygote strain in which the variant codon (R939) was replaced by the reference codon (G939) and GFP was integrated at the end of the coding region of *IMP9*; mock knockdown, a strain carrying an “empty” pMCoDel vector, in which the level of *IMP9* expression was expected to be unaffected; *IMP9* knockdown, a strain transformed with a co-deletion vector expressing an RNA that targets a portion of *IMP9* for deletion during macronuclear development. There are 90 copies of *IMP9* in the macronucleus, and likely not all copies of *IMP9* were disrupted, resulting in a “knockdown” of *IMP9* expression. Mean $\pm$ SD. $N = 3$ experiments (100 cells scored per experiment and genotype). The P values were obtained using an unpaired *t* test with Welch’s correction. A more complete set of data related to panels G–J is included in Fig. S5.

is the sought *MPD* gene in two ways. First, we tested whether the extra M row phenotype can be rescued by homologous DNA recombination using a targeting fragment designed to replace the altered base with the reference base in the *mpd-1* homozygote and also to add a sequence-encoding GFP to the 3’ end of the coding region (Fig. S5J’). Transformants in which the reference codon was restored in some of 90 macronuclear gene copies (Fig. S5J lanes 5, 6, 8 and 9) displayed a rescue of the *mpd-1* phenotype (Fig. 6, G–J and Fig. S5). Rescues were not observed in the negative control transformants in which the targeting DNA fragment contained the variant base (Fig. 6J and Fig. S5C). Next, we knocked down *THERM_00770760* expression in a WT background, using the “co-deletion” method that utilizes small RNAs to induce elimination of target genomic DNA during macronuclear development (Hayashi and Mochizuki, 2015). Clones with partial deletion of the macronuclear *IMP9* copies showed the extra M row phenotype at a low frequency, even at the permissive temperature (Fig. 6J; and Fig. S5, E and F). Attempts at isolating a clone lacking entirely intact copies of *IMP9* (based on the macronuclear phenotypic assortment) were not successful, suggesting that *IMP9* is essential and that the two *mpd* alleles studied here are either not null or specifically affect the oral patterning function of MpD. The above data taken together led us to conclude that *IMP9* is *MPD* (we will now use the *MPD* gene name as recommended [Allen et al., 1998]).

MpD is a protein with an N-terminal IBN_N PFAM domain. A BlastP search of the human proteome revealed importin-9 (IMP9Hs) as the protein with the highest homology that is distributed throughout most of the length of MpD, except for the last 110 aas. A reverse BlastP search of the *Tetrahymena* proteome using IMP9Hs returned MpD as the top match. We compared the AlphaFold2 model of MpD (AF-Q23AR9) (Jumper et al., 2021; Varadi et al., 2022) with the crystal structure of IMP9Hs (6N1Z [Padavannil et al., 2019]) (Fig. 6, F–F’). The two structures are very similar, indicating that MpD is an importin-9 (Fig. 6, F–F’).

The alpha helices of the studied importin-9 proteins are connected by loops of variable lengths, most of which are short, but a few are very long (>30 residues). The AlphaFold model of MpD shows that its long loops are conserved (Fig. 6, F–F’). The long h8loop loop of human and *Saccharomyces cerevisiae* IMP9 is important for Ran^{GTP} binding, and the h18–19loop is important for binding the well-characterized importin-9 cargo, the histone H2A-H2B dimer (Jiou et al., 2023; Padavannil et al., 2019). Conservation of h8loop suggests that MpD likely also binds Ran^{GTP}. Residues G939 and D1148, which are mutated in *mpd-1* (G939R) and *mpd-2* (D1148N), respectively, are marked in Fig. 6 F’. G939 is located in the h18loop close to the h18A helix, which is a part of the H2A-H2B-binding site of Imp9Hs. Thus, the *mpd-1* mutation may affect the binding of MpD to a positively charged cargo. D1148 is in the h19A helix. Its proximity to the positively charged residues in the long helix18B may confer structural stability in this region, and the D1148N mutation may disrupt the local fold at the C terminus of MpD.

MpD forms a gradient along M1

We edited the native *MPD* gene by adding a GFP sequence at the 3’ end of the coding region in the WT strain. In fixed interphase cells, the MpD-GFP signal was weak but above the background at two locations: (1) at the cell apex colocalizing with the ac and (2) in M1 (Fig. 8, A and A’). Remarkably, in M1, MpD formed a gradient along the M1 length, with the high point near the right (sculptured) end (Fig. 8, A and A’). We also tagged *MPD* carrying the *mpd-1* mutation, G939R. At 39°C, the M1 signal of MpD^{G939R}-GFP was greatly diminished as compared with the control MpD-GFP^{G939} (Fig. S5, G–H’), indicating that the mutant protein is either unstable or has a diminished ability to properly localize. Live total internal reflection microscopy (TIRF) confirmed a high level of MpD-GFP at the right end of M1 but also detected a weaker signal in the remaining M rows (M2 and M3) and in the posterior region of the old OA along the ribbed wall (Videos 1 and

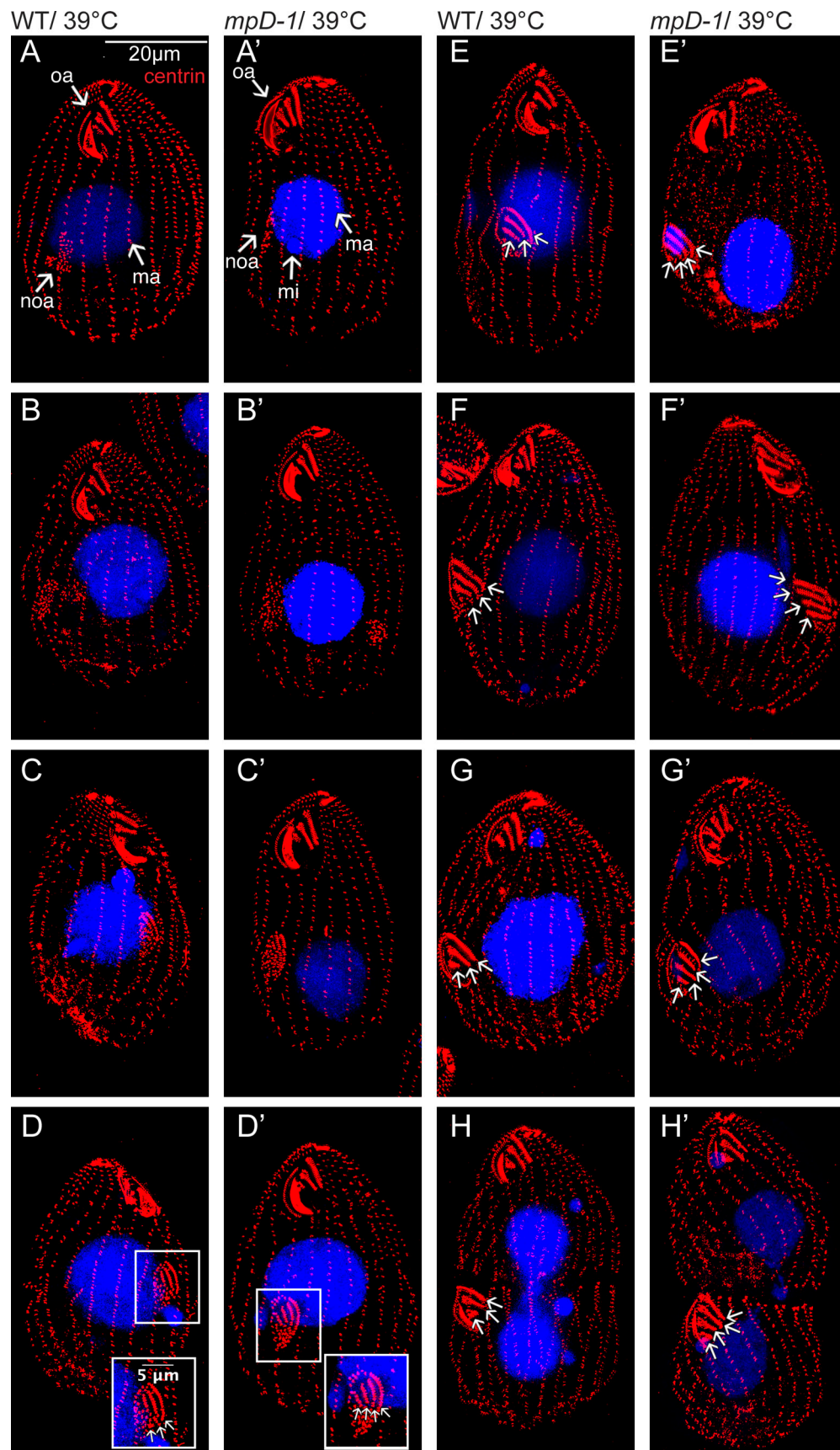


Figure 7. ***mpD-1* confers extra M row formation during development.** A comparison of the course of oral development between the WT (A, B, C, D, E, F, G, and H) and *mpD-1* homozygotes (A', B', C', D', E', F', G', and H') grown at 39°C. White arrows mark the M rows. **(A and A')** Stage 1b in the WT and 1a in *mpD-1*.

(B and B') Stage 2. (C and C') Stage 3. (D and D') Stage 4a. Note the extra M row in the mutant cell. (E and E') Late stage 4b (WT) and early stage 5a (mutant). (F and F') Stage 5b. There appears to be a delay in the formation of UM in the mutant cell. (G and G') Stage 5f in the WT and 5b in the mutant cell. (H and H') Late stage 5f. oa, mature oral apparatus; noa, new oral apparatus or OP; ma, macronucleus; mi, micronucleus.

2). MpD-GFP was also present around the perimeter of both the macronucleus and the micronucleus, indicating that MpD has retained the conserved nuclear import function (Video 3). During divisional oral development, MpD-GFP was consistently present in M1 of the old OA (Fig. 8, B-H'). In the OP, MpD-GFP was not visible during stages 1-4a (Fig. 8, B-C') and appeared close to the right end of M1 in stage 4b (Fig. 8, D and D'), which corresponds to the time of assembly of the third BB subrow in M1 and M2 and the emergence of M3. During stages 5a-5b (when the M rows undergo condensation), MpD-GFP remained prominent in M1 but was also present weakly in M2 and M3 (Fig. 8, E-G'). At stage 5a, the division boundary starts to form as a circumferential gap around the cell's equator named the "cortical subdivision" (marked "cs" in Fig. 8, E and E') (Frankel et al., 1981; Wloga and Frankel, 2012). At this stage, MpD-GFP colocalized with the terminal BB pairs of the posterior somatic half rows, which are destined to form the new ac in the posterior daughter cell (Jerka-Dziadosz, 1981; Jerka-Dziadosz et al., 2001) (Fig. 8, E-H').

Overproduction of MpD suppresses the formation of M3

We overproduced GFP-MpD by editing the 5' UTR of native *MPD* to insert the *MTTI* promoter. In the non-induced *MTTI-GFP-MPD* transgene-carrying cells, the OA consistently had a normal organization, with 3 M rows (Fig. 9, A-A"). Exposure to 2.5 µg/ml cadmium chloride (6 h) elevated the levels of GFP-MpD in the subcortical region, around the micronucleus, and inside of the macronucleus (Fig. 9, B-B"). Remarkably, 30% of GFP-MpD-overproducing cells had OAs with two instead of 3 M rows (Fig. 9, B' and E). In the OAs with two rows, M1 was longer than M2, the sculpturing pattern of M1 resembled that of aa WT M1 while M2 appeared to have a combination of features typical of WT M2 and M3 (Fig. 9 B'). Importantly, we did not observe OAs lacking all 3 M rows or having a single M row. Images of overproducing cells undergoing divisional oral development indicated that following the emergence of M1 and M2, M3 failed to form (Fig. 9, C and D). The overproducing cell shown in Fig. 9 C has an OP in stage 4b with M1 and M2 present and lacking M3. In the OPs' posterior region, there is a group of scattered BBs that are in position of M3 but had failed to assemble into a row (yellow arrow in Fig. 9 C). The cell in Fig. 9 D is in stage 5f, and the OP has only two sculptured rows (M1 and M2 based on the positions relative to the ribbed wall/UM). Thus, overexpression of GFP-MpD suppresses the formation of M3. We conclude that MpD limits row initiation after the emergence of M1 and M2. Also, these data indicate that M3 is regulated in a way that is distinct from M1 and M2.

The phenotype of double mutants supports a model that MpH promotes row elongation and MpD inhibits row initiation

The overexpression data opened a possibility that MpH, in addition to its row-elongating activity, also increases row initiation

(see Fig. 5). In an attempt to distinguish between these two activities, we examined double mutant homozygotes *mpH-1; mpD-1*. As documented above, MpD inhibits row initiation. If MpH has a significant row-initiating activity, then one would expect a normalization of the M row number in a double mutant *mpH-1; mpD-1*. Strikingly, this was not the case. At the permissive temperature, as expected, the double *mpD-1; mpH-1* mutants were phenotypically similar to the *mpH-1* single mutants (Fig. 10 I). At 39°C, the double mutants formed OAs with an increased number of M rows, which is characteristic of *mpD-1* alone, but these rows were invariably excessively short, as seen in the *mpH-1* single mutants (Fig. 10, A-C). Interestingly, the double mutants often had OAs with 5, 6, or even more M rows (a rare phenotype in the single *mpD-1* mutant), and therefore *mpH-1* enhances the *mpD-1* phenotype (Fig. 10, B, C, and I). An increased number of short M rows was observed in the OP during development (Fig. 10, D-H). These data argue against MpH having a major role in row initiation. If we assume that *mpH-1* is mainly a row elongation factor, then the double mutants present a precisely additive phenotype. The simplest interpretation based on the double mutant phenotypes is that MpH is primarily a row elongation-promoting factor, and MpD inhibits row initiation.

Discussion

During animal embryonic development, some body parts (e.g., somites, limb digits and teeth) form as series with a fixed number of elements (reviewed in Sudderick and Glover [2024]). Precise periodic patterns also exist inside cells. One example is stereocilia, mechanosensory cell projections that are organized as three rows forming a staircase on the apical surface of the vertebrate hair cells in the ear (reviewed in Barr-Gillespie [2015]). While the determination of an even number of organelles (e.g., centrioles) is controlled by a round of duplication during the cell cycle coupled with licensing mechanisms that prevent overduplication (Nigg and Holland, 2018), how precise uneven numbers of organelles are generated is unanswered. Here, we took a step toward understanding the mechanism of precise intracellular counting using the ciliate OA as a model. *T. thermophila* offers the availability of unique alleles that alter the number of oral rows, including those that change the number of M rows: *mpA* (Kaczanowski, 1975, 1976), *mpC* (Frankel et al., 1984b), *mpD* (Frankel et al., 1984a, 1984b), *mpG*, and *mpH* (Frankel, 2008); UM rows: *mum1* (Lansing et al., 1985); and both: *mpB*, *mpF*, *doa1*, and *doa2* (Frankel, 2008). The relatively recent application of comparative NGS approaches to the ciliate models (Galati et al., 2014; Marker et al., 2014) has opened the way for discovery of genes that control the cortical patterns. Here, we focused on the determination of the M row number by mapping the *mpD* and *mpH* alleles. The M rows emerge sequentially. The process can be roughly divided into two steps: (1) row emergence (stages 1-4) and (2) row differentiation (stages 5a-f). This

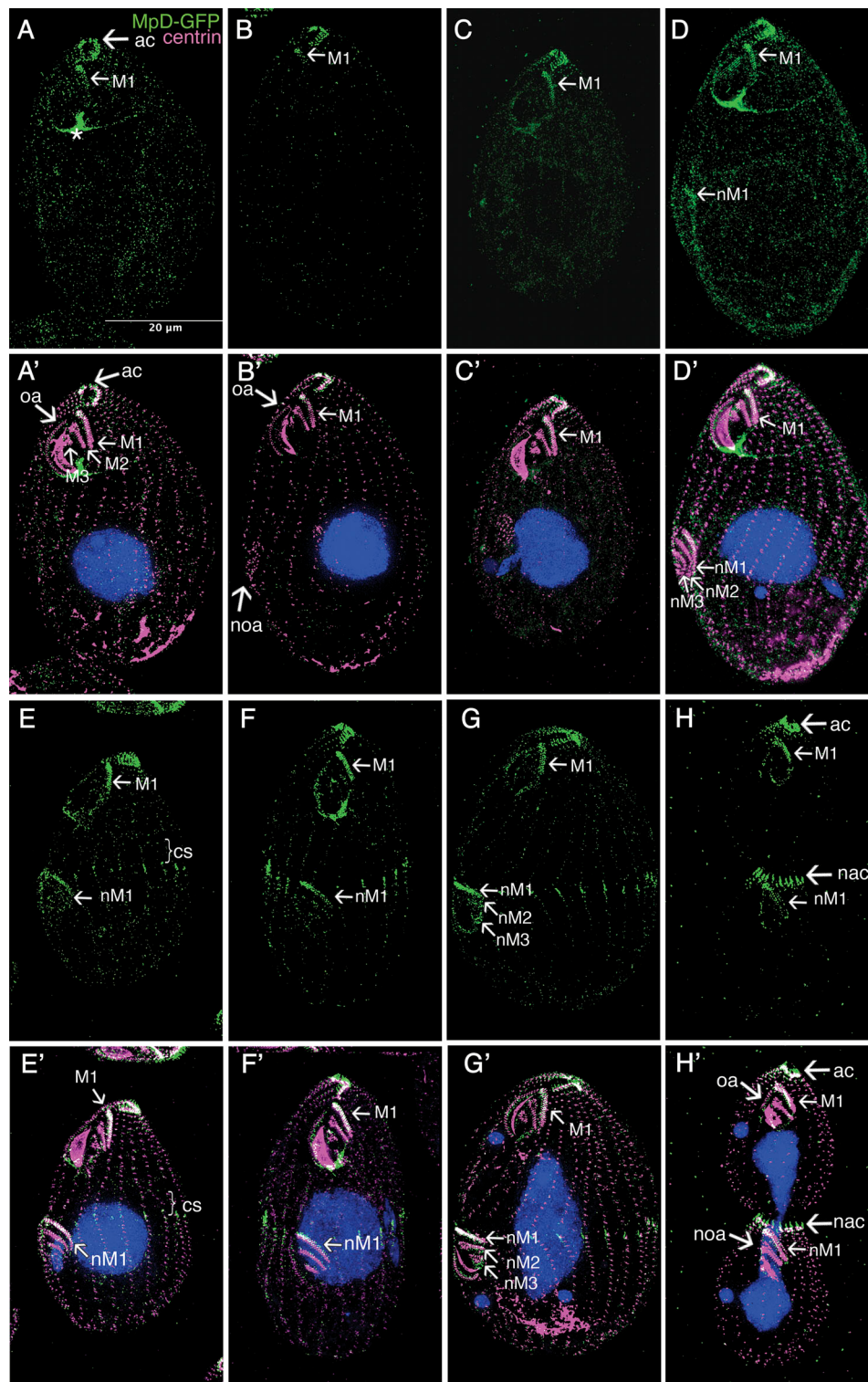


Figure 8. MpD forms a right-left gradient along M1. SR-SIM images of cells expressing MpD-GFP (green, detected by polyclonal anti-GFP antibodies) in the native locus, stained with the anti-centrin (magenta) and DAPI (blue). **(A and A')** Interphase. Note MpD-GFP at the apical cell end and along M1. The region marked with a star often binds antibodies nonspecifically. **(B and B')** Stage 1b. **(C and C')** Stage 2. **(D and D')** Stage 4a. **(E and E')** Late stage 4b. **(F and F')** An intermediate between stage 4b and 5a. **(G and G')** Early stage 5f. **(H and H')** Late stage 5f. Note the weak presence of MpD-GFP in the new M2 and M3. M(1–3), parental M rows; nM(1–3), new M rows; oa, old oral apparatus; noa, new oral apparatus or OP; ac, parental apical crown; nac, new apical crown; cs, cortical subdivision.

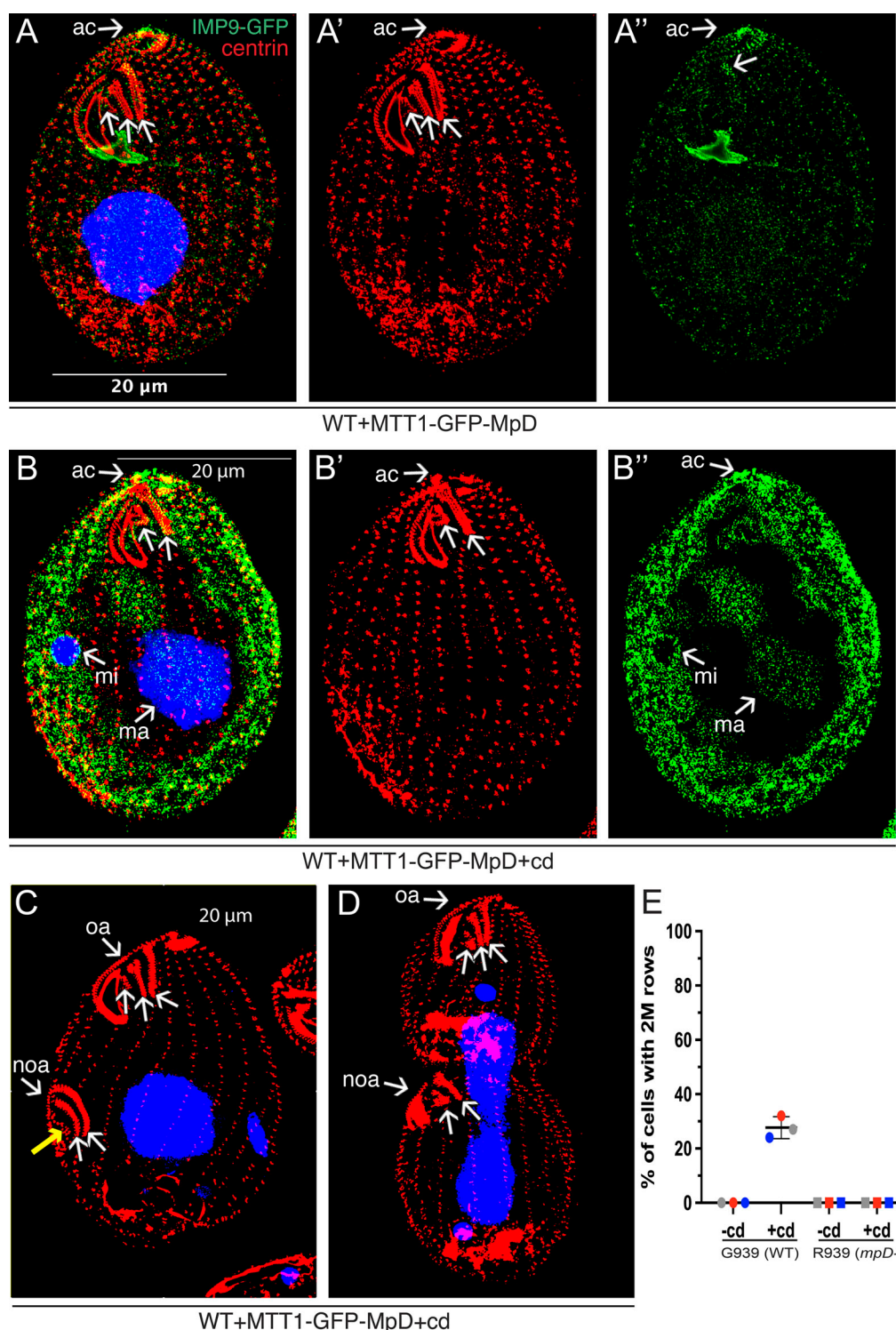


Figure 9. Overexpression of GFP-MpD suppresses the formation of M3. (A–D) SR-SIM images of cells in which the native promoter of *MPD* was replaced with the *MTT1* promoter to provide cadmium-inducible expression of GFP-MpD. Cells were grown for 6 h in the absence (A–A'') or presence (B–D) of 2.5 μg/ml cadmium chloride. Note that GFP-MpD is expressed at low level even without addition of cadmium ions due to the basal level of *MTT1* promoter activity (A–A''). In the overproducing cells (B–B''), GFP-MpD accumulates in the subcortical region, around the micronucleus, and inside the macronucleus. **(C and D)** GFP-MpD-overproducing cells during divisional oral development. The cells in C is in stage 4b; note that scattered BBs are present in the posterior region of OP (yellow arrow), but they failed to form M3. The cell in D is in late stage 5f. The new OA has 2 M instead of 3 M rows. The old oral OA has a normal 3 M organization. Thus, the defect (inability to form M3) occurs during development. **(E)** The graph quantifies the 2 M row phenotype in cells in which either the WT (G939) or mutant GFP-MpD operates under the *MTT1* promoter whose overexpression was either induced (cd) or not (–cd) by exposure to cadmium chloride (2.5 μg/ml, 6 h). The 2 M phenotype is only present in cells that overproduce a WT (G939) MpD. Mean ± SD. *N* = 3 experiments (100 cells scored per experiment). oa, mature oral apparatus; noa, new oral apparatus or OP; ac, apical crown; mi, micronucleus; ma, macronucleus. White arrows mark the M rows.

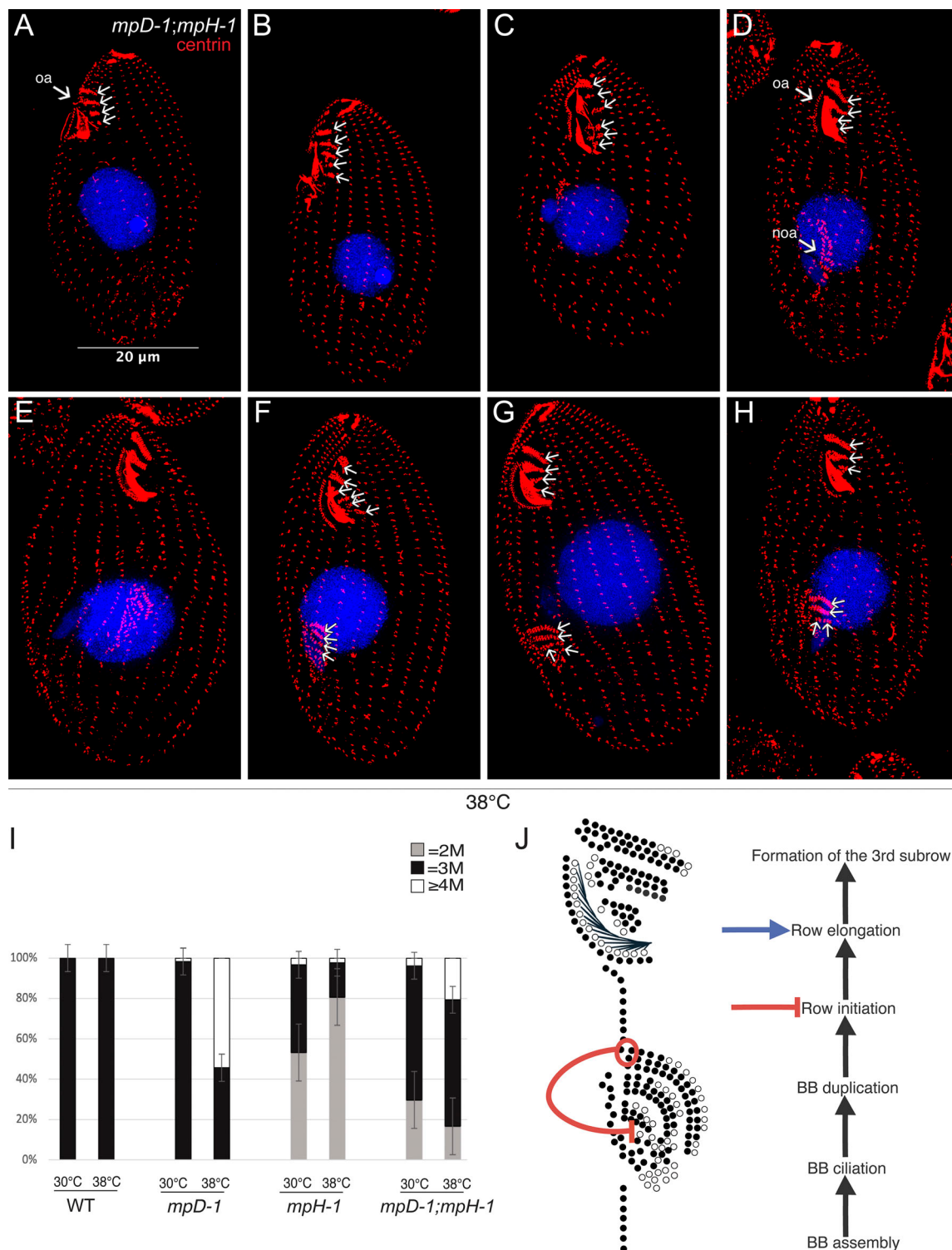


Figure 10. Phenotypes of double mutant cells *mpH-1/mpH-1*; *mpD1/mpD-1* indicate that MpH promotes row elongation and MpD inhibits row initiation. (A–H) SR-SIM images of double mutant homozygotes *mpD-1*; *mpH-1* grown at the restrictive temperature (39°C) to induce *mpD-1* phenotype expression and stained with 20H5 anti-centrin (red) and DAPI (blue). **(A–C)** Interphase cells. Note the superimposition of the short row defect (*mpH-1*) and the extra row defect (*mpD-1*). **(D–H)** Double mutant cells during divisional oral development. **(I)** Quantification of the M row configurations per OA in strains with the indicated genotypes grown at either at 30°C or 38°C. Mean $\pm$ SD. $N = 3$ experiments (100 cells scored per experiment). WT cells have exclusively a 3 M organization at both temperatures. The OAs in the *mpD-1* single mutants have either the 3 M or 4 M organization, with 4 M being highly expressed at 38°C ($54.3 \pm 9.81\%$). The 2 M phenotype is present in the single *mpH-1* mutants ($54.7 \pm 12.9\%$ at 30°C and $78.0 \pm 12.1\%$ at 38°C). The double mutants show the entire phenotypic spectrum. The frequency of cells with extra M rows increases at 38°C (2 M, $16.7 \pm 5.1\%$; 3 M, $62.7 \pm 4.9\%$; 4 or more M, $20.7 \pm 3.2\%$). **(J)** The diagram summarizes the proposed functions of MpH and MpD. The OP is in stage 4b when both MpH and MpD are active. There is a posterior-anterior gradient of maturity in the row development. New BB (open circles) form in the most posterior region. Subsequently, the BB undergo maturation, including ciliation (filled

circles), and a round of duplication to form pairs. The BB pairs align side-by-side to form rows. The row assembly can be subdivided into stages of initiation, elongation (by addition of BB pairs to the left row ends), and late maturation (formation of the third subrow). MpH promotes row elongation across the entire field. Active MpD is released from M1 (when this row reaches a certain stage of maturation), diffuses, and inhibits row initiation in the posterior region of the oral field.

division is not perfect, as the emerging rows already differ in length. Nonetheless, the two alleles that we studied here appear to affect mainly the row emergence phase. This is particularly evident for MpD, as in the *mpD* mutants, despite the change in the number of rows, the length, and even the pattern of each row tend to agree with their A/P position (Frankel, 2008; Frankel et al., 1984a, 1984b). While the *mph-1* mutants in addition to the reduction in row number, also show variable defects in row patterning (sculpturing), these are not fully penetrant and could well be indirect consequences of reduced row elongation.

We find that MpH, a TPR domain protein that is highly enriched in all M rows, acts primarily as a row elongation factor whose loss leads to shortening of all M rows and a frequent loss of the shortest and last to emerge M3. Early during development, MpH becomes enriched at the BBs close to the left margin of the OP on stage 2, the stage named the “anarchic field” due to the seemingly random positions of the BBs (Frankel, 1964). However, our data show that the early OP field is already stratified, as MpH preferentially associates with the lineage of BBs within the OP located on the left side of the primordium that are destined to become parts of the M rows. Likely, the MpH-negative BBs on the right side of the OP will form the UM. Also remarkably, MpH forms concentration gradients at the level of individual rows (across subrows) and the entire oral field (across rows), which correspond to the time of BB (and row) maturation. Possibly, the age-dependent accumulation of MpH stabilizes the forming M rows. That M1 and M2 manage to form at all in the *mph-1* mutants indicates that the allele is hypomorphic or that the function of MpH is partially provided by another MpH-like TPR protein (e.g., by THERM_00348360 that shares strong homology MpH). The selective failure of M3 assembly conferred by *mph-1* can be explained by its shortest length or late emergence (if there is a prescribed quantity of MpH available at the onset of development).

Surprisingly, MpD, a protein that limits row initiation during the time of M3 formation, is an ortholog of importin-9. Importin-9s drive nuclear import of diverse cargoes (Kimura et al., 2017), including the histone H2A-H2B dimer (Jiou et al., 2023; Mühlhäusser et al., 2001; Padavannil et al., 2019; Shaffer et al., 2023) and the actin-cofilin complex (Dopie et al., 2012). Based on the strong presence of MpD in the OA, the function in oral development likely does not directly involve nuclear trafficking. Notably, some importins have been linked to cytoplasmic functions, including the role of importin- β in assembly of the vertebrate spindle (Kaláb et al., 2006; Kalab et al., 2002; Nachury et al., 2001) and the contractile ring during cytokinesis (Beaudet et al., 2017, 2020; Ozugergin and Piekny, 2021). Several importins, including importin-9, function as cytoplasmic chaperones that prevent binding of positively charged proteins to RNAs (Jäkel et al., 2002; Padavannil et al., 2019; Yoshizawa et al.,

2018). Also, importins regulate phase separation of cytoplasmic proteins, including TDP-43 and FUS, whose mutations cause amyotrophic lateral sclerosis and frontotemporal dementia (reviewed in Springhower et al. [2020]).

Several studies have revealed potential roles of nuclear transport factors in cilia. Nucleoporins are present at the base of cilia where they interact with the transition zone proteins and contribute to gating of cilia-destined cargo (Kee et al., 2012; Takao et al., 2017; Blasius et al., 2019). Importin- β controls entry of several proteins (carrying a nuclear localization signal) to cilia, including Crumbs3 (Fan et al., 2007), KIF17 kinesin (Dishinger et al., 2010; Funabashi et al., 2017), and Gli2 (Torrado et al., 2016). Mutations in nucleoporins have been linked to ciliopathies manifested in abnormal cardiac left-right patterning (Chen et al., 2019; Fakhro et al., 2011; Marquez et al., 2021) (reviewed in Chen et al. [2023]). Our study provides further evidence for association of nuclear import factors with cilia and reveals a novel role in generation of a multiciliated pattern.

During oral development, MpD may act by binding to a (likely basic) cargo protein that activates M row initiation in the posterior region of the oral field, where new BBs continue to proliferate during stages of row emergence (stages 3–4 in Fig. 1 A). An important future goal will be to identify the cargo of MpD that acts in oral development. Among the best-studied nucleus-destined cargoes of importin-9 is the actin-cofilin complex (Dopie et al., 2012). In vertebrate multiciliated cells, the docking of BBs to the apical cell surface is driven by myosin motors that move on cortical microfilaments (Antoniades et al., 2014; Hong et al., 2015; Pan et al., 2007; Pitaval et al., 2010). Microfilaments are also important for proper orientation of BBs on the apical surface of vertebrate multiciliated cells (Herawati et al., 2016; Werner et al., 2011). Oral row initiation may require crowding of BB pairs to reach a critical concentration, which could be driven by tension generated by myosin on microfilaments. Ciliates have several isoforms of actin (Sehring et al., 2007a, 2007b; Williams et al., 2006), and microfilaments are present in the OA in association with the BBs (Hoey and Gavin, 1992). On the other hand, *Tetrahymena* has only one canonical cofilin, ADF73, does not affect the gross organization of the OA (Shiozaki et al., 2013). If actin is the oral development-relevant cargo of MpD, it is likely not in a complex with cofilin.

MpD limits row initiation at the stage of M3 emergence, in the posterior region of the OP (stage 4). This area appears to function as a “proliferative zone,” a source of new BBs that contribute to the formation of both M rows and UM. Surprisingly, MpD is highly enriched in M1 in the form of the R-L gradient along M1. The right end of M1 is the oldest part of not only of M1 but also the entire M row compartment. Previously, we localized the CdaH/Fused kinase within the same region of M1 (Lee et al., 2024). Surprisingly, while MpD is highly enriched at the right end of M1, its activity suppresses row initiation by

BBs that are located posteriorly to M3. Excessive levels of MpD suppress M3 but not M1 and M2. How can an M1-enriched protein act at the distance across the oral field? We speculate that M1, the first emerging row, serves as a platform for activation and release of active MpD that diffuses across the oral field and inhibits the row emergence in the posterior region of the OP that is enriched in the BB pairs (Fig. 10 J). In agreement with this model, in live cells, we detected a low level of MpD throughout the OA, including M2, M3, and UM. MpD accumulates in M1 at stage 4b, which is the time when M1 gains the third BB subrow (Fig. 10 J). The release of active MpD from M1 could therefore be coupled to the state of assembly of M1 (BB triplets), as a negative feedback loop. This model is inspired by the spindle assembly checkpoint, which involves assembly and release of a factor from kinetochores unattached to microtubules that is active throughout the mitotic spindle (reviewed in McAinsh and Kops [2023]). Thus, as the oldest part of the forming OA, the right end of M1 may act as an “organizer” for the oral developmental field.

Intriguingly, in addition to the OA, both MpH and MpD also localize to the cell’s apical end. The cell apex is itself asymmetric, as its main constituent is an incomplete ring of BB pairs with a gap located on the cell’s left/dorsal side (Jerka-Dziadosz, 1981; Jerka-Dziadosz et al., 2001; Wloga and Frankel, 2012). MpH and MpD may therefore act in patterning of BBs at the cell apex. While we have not detected contributions of MpH and MpD to the cell apex organization, the available alleles may be specific to oral development, or the functions of these proteins at the cell apex may be covered by other proteins, including other importins (some of which are associated with the BBs [Malone et al., 2008]) and the potential paralog of MpH, THERM_00348360.

Frankel and colleagues proposed that the developing OA is a unified developmental field in which its parts differentiate according to their positions (Frankel, 2008; Frankel et al., 1984a, 1984b). Our data lend support to this model by revealing that MpG and MpH, proteins that control the size of the field, form concentration gradients within the OA. However, the mechanism of position-dependent row differentiation, including row sizing and sculpturing, remains unknown. Speculatively, additional factors present in the primordium may act as short-range intracellular morphogens to determine the row fate. The right end of M1 could act as an “intracellular organizer” that releases such morphogens. To explore this model, future mutant screens in *Tetrahymena* would need to be focused on isolation of alleles that affect row differentiation.

Materials and methods

Tetrahymena strains and cell culture

The *T. thermophila* strains used were obtained from the *Tetrahymena* Stock Center (Cornell University, Ithaca, NY, USA; currently housed at the Washington University in St. Louis, MO, USA, <https://sites.wustl.edu/tetrahymena/>): IA305 (TSC_SD01228) (*mpD-1/mpD-1* [*mpD-1*]), IA383 (TSC_SD01231) (*mpD-2/mpD-2* [*mpD-2*]), IA483 (TSC_SD01235) (*mpH-1/mpH-1* [*mpH-1*]), CU428 (TSC_SD00178) (*mpr1-1/mpr1-1* [*MPR1*, *6mp-s*]), and

CU427.4 (TSC_SD00715) (*chx1-1/chx1-1* [*CHX1*, *cy-s*]). Cultures were propagated at 30°C in SPPA medium containing 1% proteose-peptone, 0.2% dextrose, 0.1% yeast extract, and 0.003% EDTA:ferric sodium salt (Gorovsky et al., 1975) with the antibiotic-antimycotic mix at 1:100 (30-004-CI; Corning). To express the conditional *mpD-1* and *mpD-2* phenotypes, mutant strains were grown at 38–39°C for one or more generations (3–6 h).

To generate double mutant homozygotes, double heterozygotes were obtained by standard crosses and subjected to self-crosses using strain B*VII (TSC_SD00023) to create micronuclear homozygotes. The micronuclear genotypes were determined by backcrosses and scoring of the progeny phenotypes. Pairs of heterokaryon strains with the desired micronuclear genotypes were mated with each other to produce progeny that were homozygous for both alleles in the macronucleus. The genotypes were verified by amplification of gene fragments carrying mutations and Sanger DNA sequencing.

Mapping the mutant alleles by comparative NGS

A mutant homozygous strain (IA305 or IA483) was crossed to the mapping strain CU427.4 (homozygous for *chx1-1* cycloheximide [*cy*] resistance in the micronucleus). F1 clones resistant to *cy* were transferred daily on nonselective medium for ~10 days; subclones were made and screened for *cy* sensitivity. A single highly fertile *cy*-sensitive F1 clone was subjected to a self-cross strategy of uniparental cytogamy (Cole and Bruns, 1992) using B*VII, and the F2 progeny clones were selected with *cy*. The phenotypes of F2 clones (at 39°C in the case of *mpD-1*) were determined by immunofluorescence using 20H5 anti-centrin. 40 F2 progeny clones showing the mutant phenotype and the same number of clones showing the WT phenotype were pooled. Total genomic DNA was extracted from the two F2 pools as described (Jiang et al., 2017) and subjected to sequencing on the Illumina HiSeq X instrument to generate paired reads of 150 bp in length at the 90x genome coverage. The raw NGS data are available at the SRA database (Bioproject: PRJNA1167461). The identification of variants linked to the mutant phenotypes was done using the MiModD suite (version 0.1.8; <https://sourceforge.net/projects/mimodd/>) and the ACCA protocol (Jiang et al., 2017). The sequence variants were mapped to the micronuclear chromosomes (GenBank GCA_000261185.1 [Hamilton et al., 2016]), and the linkage scores were calculated on the basis of contrast in allelic composition between the WT and mutant F2 pool. In addition, the *mpD-1* variants were also mapped onto the macronuclear reference genome of the WT strain SB210 (GenBank GCA_000189635 [Eisen et al., 2006]). These analyses showed a peak in the ACCA signal around 20 Mb of chr3 for *mpD-1*, and around 7.5 Mb of chr1 for *mpH-1*, prompting consideration of homozygous variants present in these regions that were predicted to affect gene products: THERM_00770760/IMP9 for *mpD-1* and THERM_00564170 (tentatively named TTC6) for *mpH-1*. Another variant in THERM_00770760/IMP9 was detected by Sanger DNA sequencing in the strain homozygous for *mpD-2* (IA383).

To confirm the predicted splice site mutation in the mRNA of THERM_00564170 in the *mpH-1* homozygotes, total RNA was

extracted from CU428 and IA483 strains using the Purelink RNA Mini Kit (Invitrogen). cDNA was synthesized using the AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies) with an Oligo(dT) primer. PCR was performed with Hercules II Fusion DNA Polymerase (Agilent Technologies) using *THERM_00564170*-specific primers: 5'-GTTGATGAGTAA GCTTTAGCAGATATCT-3' and 5'-ATCAAGCTTGCCATCCGC GGTAACTCTTATTCTTTTTTATAGTTTGTCTAG-3'.

Genome editing in the macronucleus

To test whether *THERM_00770760/IMP9* is the locus of *mpD* alleles, we created a strain with a deletion within the macronuclear *THERM_00770760/IMP9* gene using the RNA-based co-deletion method (Hayashi and Mochizuki, 2015). A 980-bp genomic fragment of *THERM_00770760/IMP9* was amplified using primers 5'-CAGTTCTCATCAAGTTGTAATGCTAAATGCGG CCGCGCAAATGCGTATTACATAGGTGCT-3' and 5'-GGACTCTTT ATTGTTATCATCTTATGACCGCGCGCTGCCATAATTCTACTC CATCGAAAAC-3' and subcloned into the co-deletion vector pMCoDel using the NotI site. The resulting pMCoDel-IMP9 plasmid was introduced by biolistic bombardment into conjugating B2086 and CU428 cells, and transformants were selected with 100 µg/ml pm as described (Hayashi and Mochizuki, 2015).

To test whether the *mpD-1* phenotype can be rescued by replacing the mapped variant in *IMP9*, we constructed plasmids carrying a portion of *IMP9* covering the variant site and containing either the reference or the altered base, followed by the sequence-encoding GFP, a stop codon TGA, a portion of the 3'UTR of *BTUI*, *neo5* selectable marker, and a portion of the 3' UTR of *IMP9*. The two ~1.1-kb flanking fragments of *IMP9* were amplified with the primer pairs: 5'-CTATAGGGCGAATTGGAG CTCTCTTGGTGGTTCTGCTGATC-3', 5'-ATCAAGCTTGCCATC CGCGGGTATATTTATTAAGAATGTAGCTGTTTC-3' and 5'-GCT TATCGATACCGTCGACCGTTTAAATATAATGAATGAGTCTTA TT-3', 5'-AGGGAACAAAAGCTGGGTACGGCTGCTGATAATGAA GAATAGA-3'. The portion of the resulting plasmid ending with fragments homologous to *IMP9* was introduced into the *mpD-1* homozygous mutant cells (IA305) using biolistic bombardment, and transformants were selected with pm. The allelic ratio between the reference and altered variant base was determined by PCR amplification using primers 5'-GCTTCTTGG TGGTCTGCTGAT-3' and 5'-CTAATCAATGTACTTAAATTTAAGTGGAGCAC-3' and digestion with MnlI restriction enzyme, as the *mpD-1* allele-associated variant eliminates this restriction site.

To tag *IMP9/MPD* at the native locus, a sequence-encoding GFP was incorporated at the 3' end of the *MPD*-coding region along with the *BTUI* 3'UTR acting as transcription terminator and *neo5*. The required targeting plasmid (pIMP9-GFP) was made by amplifying two ~1.1-kb fragments of *MPD* (using CU428 genomic DNA as template) with the primer pairs: 5'-CTATAG GGCGAATTGGAGCTCTTCTTGGTGGTTCTGCTGATC-3', 5'-ATC AAGCTTGCCATCCGCGGGTATATTTATTAAGAATGTAGCTGT TC-3' and 5'-GCTTATCGATACCGTCGACCGTTTAAATATAAT GAATGAGTCTTATT-3', 5'-AGGGAACAAAAGCTGGGTACGGCT GCTGATAATGAAGAATAGA-3' that were cloned on the sides of

pGFP-BTUI-*neo5* (Jiang et al., 2017). To tag MpD with GFP-mNeonGreen, the plasmid pIMP9-GFP-mNeon was made by amplifying the mNeonGreen sequence with primers: 5'-GGC ATGGATGAACATAACAAACGCGTCATGGCAAGCTTGATGGTT TCT-3' and 5'-TTCGCTTACGGATCCTCAGACGCGTAAGTTGT-3' and cloning into the MluI site of pIMP9-GFP. The gene targeting portions of the resulting plasmids were introduced by biolistic bombardment (Cassidy-Hanley et al., 1997) into WT (CU428) cells, and transformant clones were selected with 100 µg/ml pm. Transformant clones were grown in SPPA with and pm concentration incrementally raised to 1,000 µg/ml to promote allele replacement by phenotypic assortment, and assorted clones were maintained at 250 µg/ml.

For overexpression of WT MpD, the *MPD* locus was edited by homologous DNA recombination to insert the *MTT1* promoter, followed by the sequence-encoding GFP (Shang et al., 2002) at the 5' end of the coding region, with a linked *neo5* marker. To construct the needed targeting fragment, pNeo5-MTT1-GFP was used as a base plasmid (Jiang et al., 2019). Two ~1.3-kb fragments of *MPD* were amplified with the primer pairs 5'-GCATGG ATGAACATAACAAACGCGTCATGGACTCCAATTCAGTTAGTT AGC-3', 5'-AGGGAACAAAAGCTGGGTACGGGGCCAGGAATC AAAGGAGTGAGTGAG-3' and 5'-ATAGGGCGAATTGGAGCTCCC ACTAAATCTCTGCATCATGC-3', 5'-TAGAGCGGCGCCACCGC GGACTTATTAGTTTTGTTTAATTATTCTTCTTTGT-3' and subcloned into pNeo5-MTT1-GFP. The gene-targeting portion was introduced into CU428 (WT) and IA305 (*mpD-1*) strains using biolistic bombardment, and transformants were selected using pm as described above. The resulting strains were used to overproduce either the WT or *mpD-1* version of GFP-MpD by exposing cells to 2.5 µg/ml cadmium chloride for 6 h.

Another plasmid was constructed for tagging the end of the coding region of *THERM_00564170*, named pTTC6-GFP-mNeon. The plasmid contains sequence elements in the following order: a portion of the coding region of *THERM_00564170* amplified with primers 5'-AGAGCTTGACGGGGAAAGCCGGCAGGGTATTGAAGG CATCTGCT-3' and 5'-ATCAAGCTTGCCATCCGCGTTAATCTTTA TTCTTTTTTATAGTTTGTCTAG-3', GFP, mNeonGreen, TGA stop codon, 3' UTR of *BTUI*, *neo5*, and a portion of the 3' UTR of *THERM_00564170* amplified with primers 5'-TATCAAGCTTAT CGATACCGTCGACGAGTGGGTACGATAAAATGAGAAAAT-3' and 5'-AAGATTTAAATAAGCTCCTCTGAGCTCACCCTTTCTCTGAA ATAGCCCT-3'. The plasmid was digested with NaeI and SacI to release the targeting portion and used for biolistic transformation of the *mpH-1* mutant homozygous strain IA483 to test whether the mutant phenotype can be rescued by replacing the variant linked to *mpH-1*. The same approach was used to tag the coding region of *THERM_00564170* in the WT background for imaging of MpH.

Microscopic imaging

T. thermophila cells were immunostained using the rapid method described by Gaertig et al. (2013), with minor modifications. On a 22 × 22-mm cover glass, 20 µl of cell culture was mixed with an equal volume of 0.25% Triton X-100 and 1% paraformaldehyde in the PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, and 2 mM MgSO₄, pH 6.9). After air drying at 30°C, the cover glass was washed three times with PBS and incubated overnight

with primary antibodies diluted in PBS supplemented with 3% BSA fraction V and 0.01% Tween-20. The primary antibodies used were polyclonal rabbit anti-GFP antibodies (600-401-215; at 1:800 dilution; Rockland Immunochemicals) and monoclonal mouse anti-centrin 20H5 (41624; 1:200–1:400; EMD Millipore). The secondary antibodies used were goat-anti-rabbit-Ig-Cy3, goat-anti-mouse-Ig-FITC, or donkey-anti-mouse-Alexa Fluor 647 (115-095-46; 111-165-003; 715-605-150; 1:200; Jackson ImmunoResearch). The nuclei were stained with 0.5 µg/ml DAPI (AdipoGen). The stained cells were mounted in 90% glycerol, 10% PBS, and 100 mg/ml DABCO (Sigma-Aldrich). Confocal images were acquired using a ZEISS LSM 710 microscope, equipped with a 63×/1.40 oil DIC M27 objective and Argon/2 laser. SR-SIM imaging was conducted on a ZEISS ELYRA S1 microscope, equipped with a 63× NA 1.4 Oil Plan-Apochromat DIC objective and high-power solid-state lasers (405, 488, and 561 nm). The images were processed using ZEN 2011 (ZEISS) and Fiji/ImageJ software (Schindelin et al., 2012). TIRF imaging of partially immobilized live cells was conducted at the room temperature as described (Jiang et al., 2015; Lechtreck, 2016) using a Nikon Eclipse Ti-U inverted microscope, equipped with a 60×/1.49 objective lens and a 40 mW 488-nm diode laser (Spectra-Physics).

Statistical analyses

Quantifications were conducted using data from three independent biological replicates. Statistical significance was assessed using Prism 10 (GraphPad). The sample sizes and the types of statistical tests used are described in the figure legends. The normality of data distribution was assessed using the Shapiro–Wilk test in GraphPad Prism. When the Shapiro–Wilk test was inconclusive, the data were assumed to have a normal distribution.

Online supplemental material

Fig. S1 d shows the correct mapping of *mpH-1* to *THERM_00564170*. Fig. S2 is an alignment of aa sequences of MpH and its ciliate orthologs showing conservation of the protein region affected by *mpH-1*. Fig. S3 contains the images of *mpD-1* mutant cells undergoing OR in the growth medium at the restrictive temperature. Fig. S4 shows the images of dividing *mpD-1* mutant cells at the restrictive temperature. The use of GFP-MpH as an M row marker confirms that *mpD-1* mutants assemble extra M rows at the restrictive temperature. Fig. S5 supports the correct mapping of *mpD-1* to *IMP9*. Videos 1, 2, and 3 are TIRF videos of live cells expressing MpD-GFP; document MpD-GFP enrichment along M1 (Videos 1 and 2), its weak presence in M2 and M3 (Video 2), and its presence around the perimeter of both the macronucleus and micronucleus (Video 3).

Data availability

The raw NGS data have been deposited at the public SRA database (Bioproject PRJNA1167461). All newly constructed strains will either be available through the *Tetrahymena* Stock Center or provided directly by J. Gaertig.

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Supplemental material

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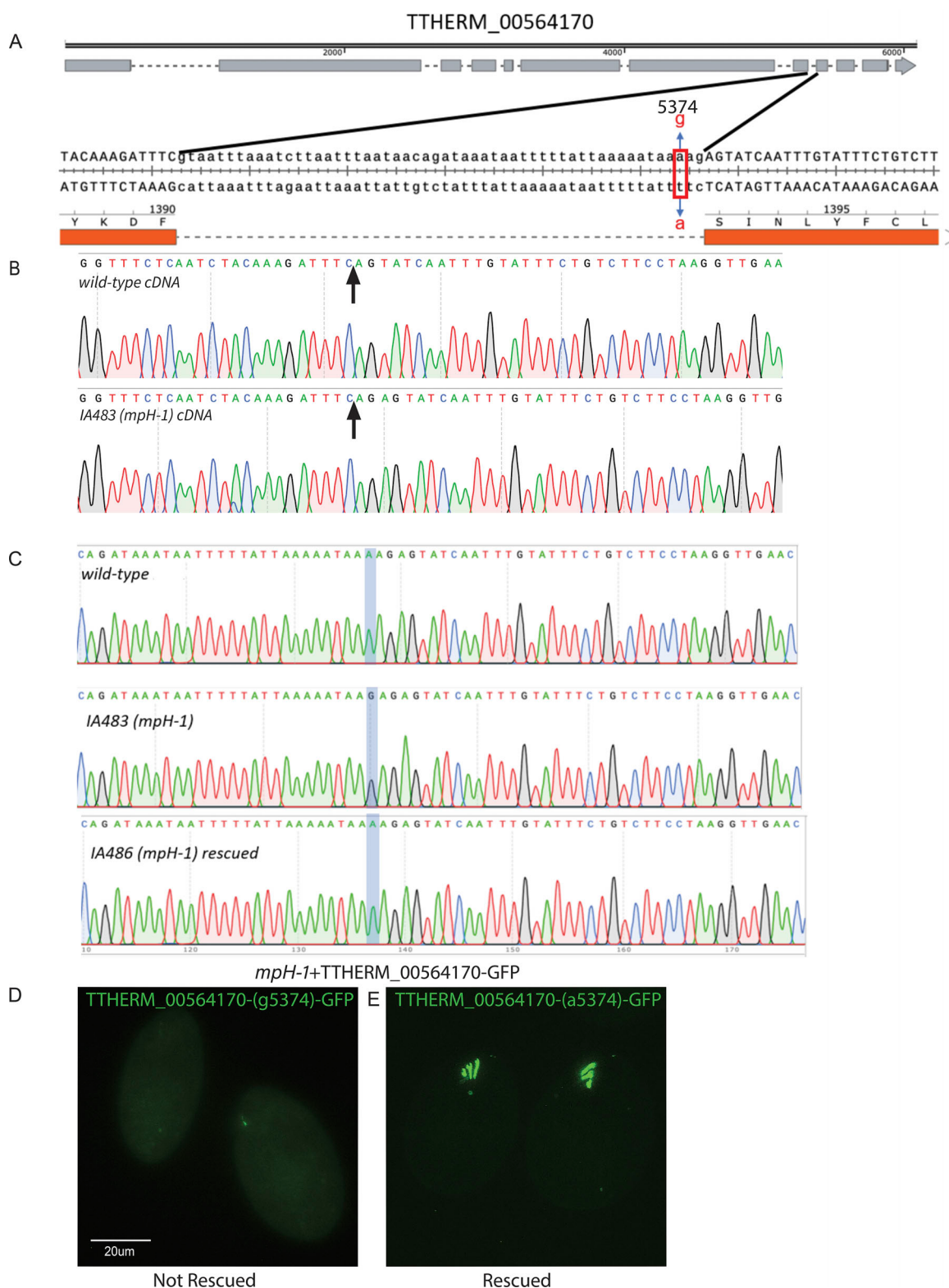


Figure S1. **mpH-1 is a gained splice site mutation in TTHERM_00564170.** (A) The diagram shows the locations of predicted introns and exons in TTHERM_00564170. Below is the sequence of intron 8 and the location of the mpH-1 G/A substitution, 2-bp upstream of the predicted 3' splicing junction. (B) Sequences of fragments of RT-PCR products amplified using TTHERM_00564170-specific primers positioned outside of exon 8. Arrows mark the positions at which exon 8 sequence transitions into exon 9 sequence following splicing of intron 8. Note the presence of two extra bases in the mutant sequence. (C) Sequencing of a portion of TTHERM_00564170 gene in the WT (top), mpH-1 homozygote (IA486 strain, middle), and a rescued clone after transformation with the targeting fragment. (D and E) SR-SIM images of cells from transformants clones with GFP-mNeon-neo5 sequence integrated at the end of the coding region that have the either variant G base and the mutant phenotype (D) or the WT A base at the variant site and the WT (rescued) phenotype (E). Note a strong signal of MPH-GFP-mNeonGreen in the phenotypically rescued transformant.

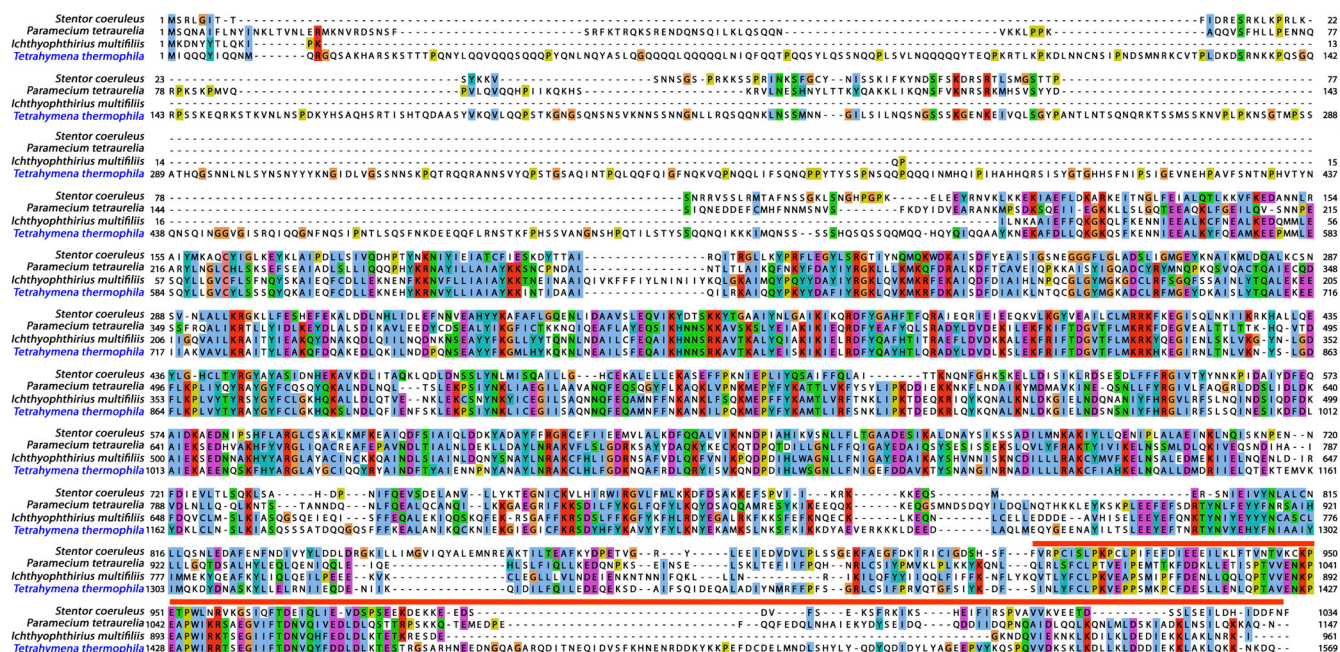


Figure S2. The C-terminal region of MpH that is altered by the *mpH-1* mutation is conserved in other ciliates. A multiple sequence alignment of THERM_00564170/MpH with its orthologs in *Ichthyophthirius multifiliis* (IMG5_005100), *P. tetraurelia* (GSPATT00023390001), and *S. coeruleus* (Ste-Coe_10711). The C-terminal region, altered and truncated in the *mpH-1* mutant, is marked with the red line above the alignment.

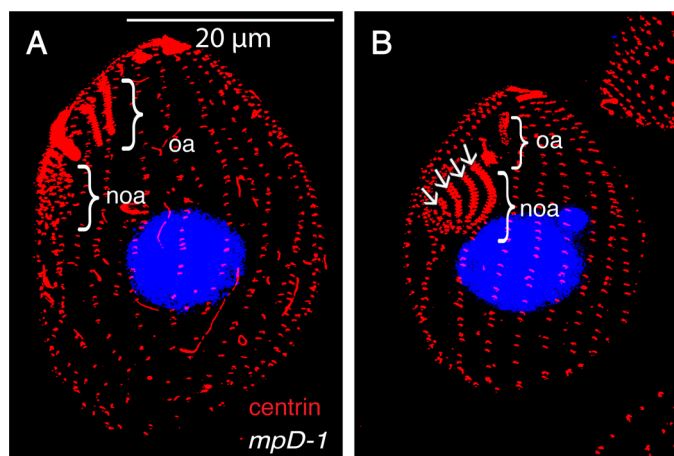
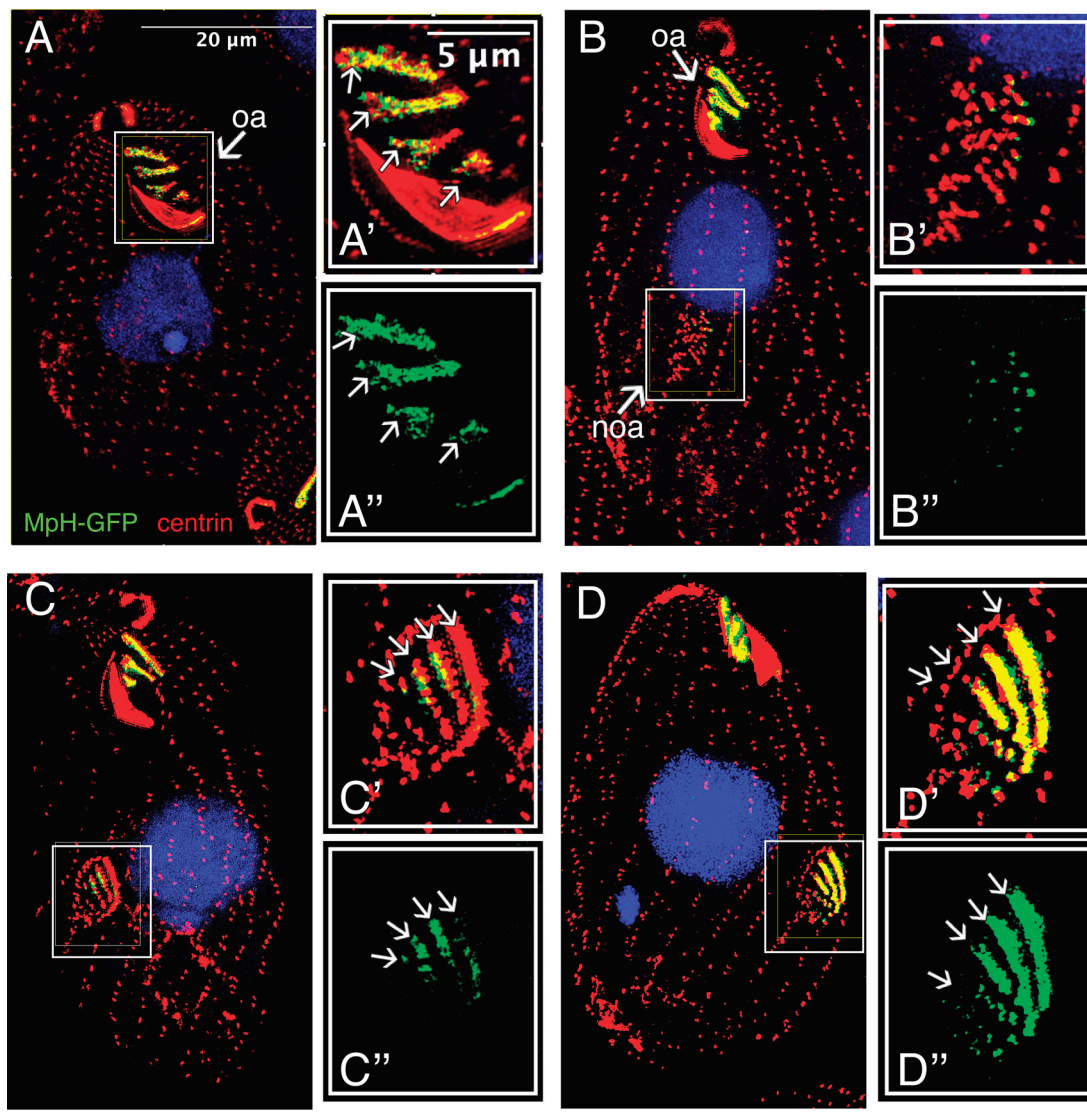


Figure S3. *mpD-1* homozygotes frequently undergo OR. (A and B) SR-SIM images of *mpD-1* homozygotes at 39°C that undergo OR. Note the formation of 4 M rows in the cell shown in B (stage 4b).



mpD-1 + MpH-GFP / 39°C

Figure S4. ***mpD-1* mutants develop extra M rows at the restrictive temperature.** (A–D) SR-SIM images of *mpD-1* homozygotes that express MpH-GFP-mNeonGreen (green) labeled with the anti-centrin antibody (red) and DAPI (blue). Insets show either the old OA (A–A'') or the OP region (B–D'') at a higher magnification. (A–A'') Interphase. (B–B'') Stage 2. (C–D'') Stage 4b. Note the presence of 4 MpH-positive rows.

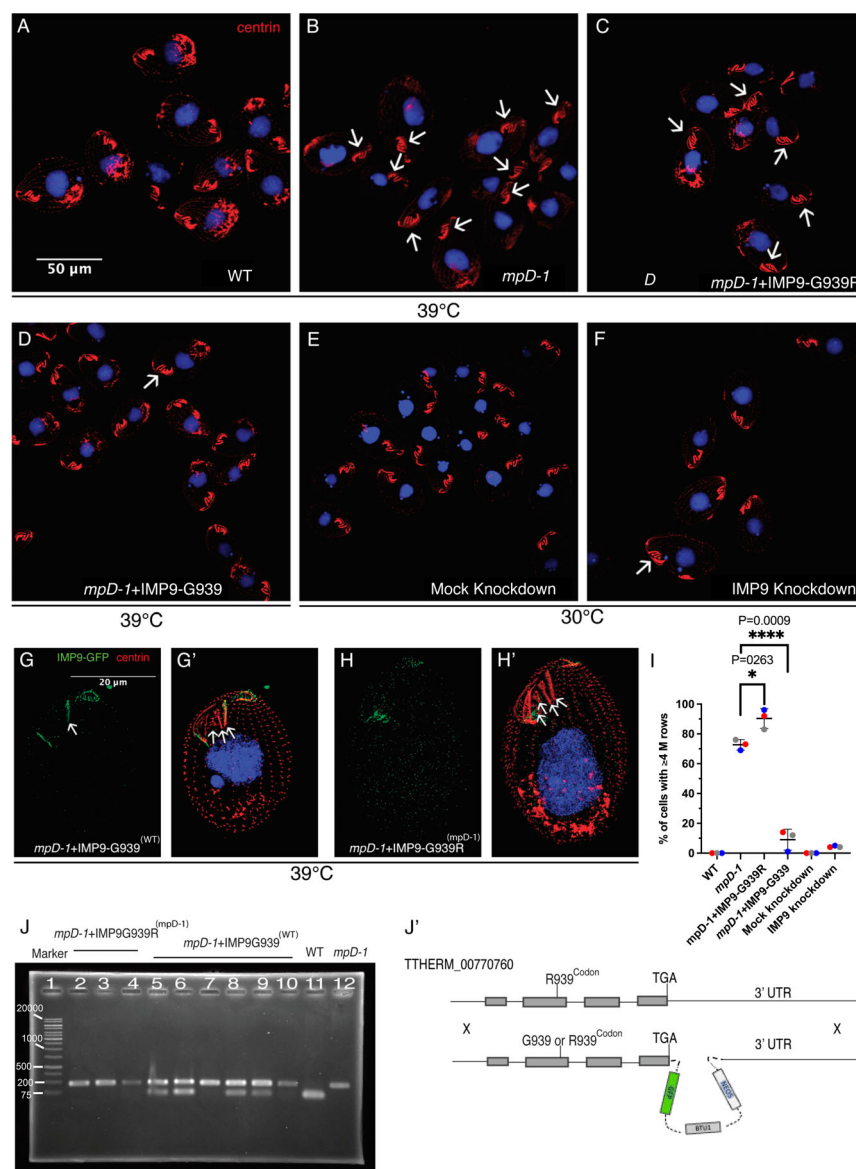


Figure S5. Genetic tests confirm that *THERM_007700760/IMP9* is MPD. (A–F) Cortical organization in cells stained with the anti-centrin antibody (red) and DAPI (blue) imaged using confocal microscopy. The genotypes are as indicated. White arrows point to OAs with a 4 M organization. The cells shown in C are *mpD-1* mutant homozygotes transformed with a targeting fragment that tags the variant allele of *IMP9* with GFP-neo5 sequence. In D, the *mpD-1* homozygotes were transformed with a targeting fragment that replaced the variant base with the WT base and added the GFP-neo5 sequences. Note fewer OAs with 4 M rows. (E and F) A knockdown of *THERM_007700760/IMP9* partially phenocopies *mpD-1/39°C*. (E) Cells (grown at 30°C) that were subjected to a mock knockdown using an empty “co-deletion vector” that does not mediate production of small RNAs targeting *IMP9*-coding region for elimination. (F) Cells (grown at 30°C) in which a portion of the coding region of *IMP9* was eliminated using the co-deletion method that produces a knockdown. (G and H') SR-SIM images of cells expressing IMP9-GFP that where the IMP9 portion is either WT (G939) (G and G') or has the *mpD-1*-linked mutation G939R (H and H'), grown at 39°C. Note that the mutant protein is depleted from M1, suggesting that the mutation reduced the stability or cortical targeting of MpD. (I) Quantification of the extra M row phenotype in various genetic backgrounds. The following genotypes were scored: WT; *mpD-1*, mutant homozygous (strain IA305); *mpD-1+IMP9-G939R*, an *mpD-1* homozygous strain carrying the *mpD-1*-linked variant (G939R codon substitution) and GFP-neo5 integrated at the end of the coding region of IMP9; *mpD-1+IMP9-G939*, an *mpD-1* homozygous strain in which the mutant codon (R939) was replaced by the WT codon (G939) and GFP-neo5 was integrated at the end of the coding region of IMP9; mock knockdown; cells transformed with a negative control empty pMCoel (co-deletion) vector; and *IMP9* knockdown, cells transformed with a co-deletion vector pMCoel expressing an RNA that targets a portion of *IMP9* gene for deletion during macronuclear development. There are 90 copies of IMP9 in the macronucleus, and likely not all copies were eliminated, resulting in a knockdown of *IMP9* expression. Mean ± SD. N = 3 experiments indicated by different symbol colors (100 cells scored per experiment and genotype). The P values were obtained using an unpaired *t* test with Welch's correction. (J) A gel image showing PCR products of amplification of a portion of *IMP9* after digestion with MnlI restriction endonuclease that distinguishes between the WT and *mpD-1*-linked alleles. Genomic DNAs were purified from a strain IA305 (*mpD-1* homozygous) subjected to gene replacement using a fragment of *IMP9* that contained either the *mpD-1*-linked variant codon (G939R) (lanes 2–4) or the reference codon G939 (lanes 5–10). Lane 11, WT genomic DNA and lane 12, *mpD-1* mutant genomic DNA (strain IA305). MnlI distinguishes between the *mpD-1*-linked variant and reference *IMP9* allele. Note a partial (lanes 5, 6, 8, and 9) replacement of the variant base with the reference base (see Fig. 6 I). (J') The diagram shows the strategy that used homologous DNA recombination to tag the 3' end of the coding region of *IMP9* in the mutant homozygous strain IA305, using fragments that encode either the reference (G939) or *mpD-1*-linked variant codon R939.

Video 1. **TIRF imaging of MpD-GFP.** MpD-GFP was expressed in a WT strain of *Tetrahymena*. A portion of a partially immobilized cell is visible. The apical end of the cell is on the left side of the video. The field of view shows the mature OA. Arrowheads point to the ac and M1 row where MpD-GFP is highly enriched.

Video 2. **TIRF imaging of MpD-GFP.** MpD-GFP was expressed in a WT strain of *Tetrahymena*. A portion of a partially immobilized cell is visible. The field of view covers the mature OA. The anterior end of the cell is on the right side of the video. Arrowheads point to MpD-GFP signals that colocalize with the M rows: M1, M2, and M3. A weak signal is also present along the UM.

Video 3. **TIRF imaging of MpD-GFP.** MpD-GFP was expressed in a WT strain of *Tetrahymena*. A portion of a partially immobilized cell is visible. The focal plane is inside the cell body. The arrowheads show MpD-GFP that colocalizes close to the periphery of the micronucleus (mi) and macronucleus (ma).