Sulfatase modifying factors control the timing of zebrafish gastrulation morphogenesis

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ABSTRACT

To shape the emerging body plan, morphogenetic cell movements must be coordinated not only in space, but also in time. Convergence and Extension (C&E) movements that elongate the anteroposterior axis initiate with precise timing during vertebrate gastrulation, but the mechanisms controlling their onset remain unknown. We examined this question using zebrafish embryonic explants that faithfully recapitulate C&E cell movements and their precise timing in culture, in isolation from other gastrulation movements. We determined that new transcription is required at gastrulation onset for C&E in explants and identified *sulfatase modifying factor 2* (*sumf2*) as a candidate 'trigger' of C&E onset. *sumf2* encodes for pFGE, the paralog and inhibitor of the Formylglycine Generating Enzyme (FGE, encoded by *sumf1*), which is the key activator of all sulfatase enzymes. In zebrafish embryos and explants, *sumf1* expression declines just as *sumf2* is expressed, leading to decreased sulfatase activity and increased sulfation of their heparan sulfate proteoglycan (HSPG) substrates. We found that overexpression of *sumf1* and *sumf2* causes delayed or precocious C&E onset, respectively, whereas mutations in *sumf1* and *sumf2* shift C&E timing in the opposite direction. We further identified Sulf1, an extracellular sulfatase that modifies HSPGs, as the key effector by which *sumf1* and *sumf2* control C&E timing. Accordingly, reduced or increased levels of sulfated heparan sulfate similarly shift C&E onset and suppress *sumf1* and *sumf2* mutant phenotypes. Together, our work supports a model in which *sumf2*

expression at zebrafish gastrulation onset reduces sulfatase activity, rewriting HSPG sulfation patterns to promote and/or permit C&E morphogenesis.

Key words: Sumf1, Sumf2, Sulf1, zebrafish, convergence and extension, HSPGs, gastrulation

INTRODUCTION

Morphogenetic cell movements must be coordinated not only in space, but also in time to properly shape the embryonic body plan. Indeed, changes in the timing of developmental events, termed heterochrony, can underlie malformations in individuals and fuel evolutionary change in populations (*1, 2*). One striking example of morphogenetic timing is the onset of gastrulation cell movements that form the primordial germ layers and shape them into the nascent embryonic axes. In many species, multiple gastrulation movements occur simultaneously and/or in rapid succession, such that the absolute timing of one process is necessary to preserve its timing relative to the others. For example, many teleost fish and amphibian embryos exhibit epiboly (which thins and spreads the epiblast), internalization (which brings mesoderm and endoderm germ layers inside the embryo), and convergence & extension (C&E) (which elongate the anteroposterior axis) movements simultaneously. The onset of epiboly in zebrafish is thought to result from a rapid fluidization of the epiblast, a consequence of cell rounding during meta-synchronous cell cleavages (*3, 4*). Initial internalization of mesoderm and endoderm cells may be triggered by a threshold level of Nodal signaling activity (*5*), which promotes cell protrusions that “un-jam” cells to enable them to ingress at the margin (*6*) or ectopically (*7*). Once leader cells ingress at the margin, “followers” in adjacent spatial domains subsequently internalize in a temporally ordered fashion according to their expression of *hox* genes (*8*), similar to a mechanism reported during chick gastrulation (*9*). However, mechanisms controlling the timing of C&E morphogenesis remain elusive.

C&E movements simultaneously narrow and elongate embryonic tissues via polarized cell rearrangements, providing the major driving force of anteroposterior (AP) axis extension and, in many species, neural tube closure (*10-13*). C&E are driven by polarized cell behaviors including mediolateral (ML) cell elongation and alignment, ML-biased cell protrusions, and polarized contraction of cell interfaces by which cells exchange

57 neighbors to form a longer and narrower array (*14, 15*). Vertebrate C&E movements and/or their underlying ML
58 intercalation behaviors begin suddenly at mid-gastrulation (*16-18*). In zebrafish, this is marked by a switch in
59 the trajectory of cell movements toward the embryo's dorsal side at ~75% epiboly (~8 hours post fertilization
60 (hpf)) (*18, 19*). Evidence suggests that these movements result not from chemotaxis (*20*) but instead from
61 spatially restricted cell-cell interactions, the domains of which are defined by morphogen signaling gradients
62 (*21-23*). The signature ML polarity of vertebrate C&E behaviors is under control of planar cell polarity (PCP)
63 signaling, which orients cell behaviors with respect to the embryonic axes (*24, 25*). This results from the
64 polarized membrane localization of core PCP signaling components (*26-29*), which directly precedes the onset
65 of C&E cell behaviors (*22, 30*). The precision and coordination of these events imply the existence of a timing
66 cue that determines the onset of C&E cell behaviors. Several morphogen signaling pathways are known to
67 govern C&E, with BMP preventing C&E of the ventral mesoderm (*21, 31*) and Nodal and FGF signaling
68 promoting C&E dorsally (*22, 32-36*). However, all these pathways are active hours before C&E begins at mid-
69 gastrulation, suggesting that their activity is not responsible for the timing of C&E onset. Indeed, although
70 Activin (which signals through the Nodal pathway) was sufficient to induce C&E of *Xenopus* animal cap
71 explants, its timing remained constant regardless of when Activin was applied (*37*). This suggests that the
72 timing mechanism functions in parallel with known C&E regulators, but its molecular basis is unknown.
73 Mechanisms controlling the timing of other early embryonic events has been extensively studied, including
74 early cell cycles and zygotic genome activation (ZGA). For example, early cell cleavages are controlled by
75 cyclical expression and degradation of maternally expressed Cyclins (*38*). After a set number of cell divisions,
76 ZGA is triggered by a threshold nuclear/cytoplasmic (N/C) ratio (*39, 40*) (*40-42*), which is thought to function
77 through titration of histones and other nuclear factors (*41-44*). In amphibian embryos, gastrulation timing was
78 linked not to fertilization or ZGA , but the first embryonic cleavage (*45, 46*), and this timing is reportedly under
79 predominantly cytoplasmic control (*45, 47-49*). However, the onset of gastrulation morphogenesis is
80 unchanged in embryos with varying cell size and cell cycle length (*49-52*), making mechanisms that count cell
81 cycles or measure N/C ratios unlikely regulators of C&E timing. Zebrafish gastrulation morphogenesis requires
82 zygotic transcription (*53*), but its timing is uncoupled from that of ZGA, instead supporting a model by which
83 new gene expression triggers C&E at a specific time.

84 Using a reductive zebrafish embryonic explant model in which C&E are isolated from the other gastrulation
85 cell movements, we determined that new transcription is required at gastrulation onset for C&E to occur, and
86 that *sulfatase modifying factor 2* (*sumf2*) is initially expressed during this critical window in both explants and
87 intact embryos. *sumf1* and *sumf2* encode Formylglycine Generating Enzyme (FGE) (*54-57*) and its antagonist
88 and paralog pFGE (*58-60*), respectively, a pair whose balance determines the activity of every sulfatase
89 enzyme in the body (*61-64*). *sumf1* is maternally expressed, and its transcript abundance drops just as *sumf2*
90 is expressed at gastrulation onset, inverting *sumf1/sumf2* levels and altering sulfation in the embryo. We show
91 that overexpression of *sumf1* and *sumf2* causes delayed or precocious C&E onset, respectively, in both
92 explants and embryos, whereas mutations in *sumf1* and *sumf2* shift C&E timing in the opposite direction. We
93 further identified Sulf1 as a key sulfatase by which Sumf1 and Sumf2 modify C&E timing. Reduced or
94 increased levels of sulfated heparan sulfate, the predominant substrate of Sulf1 (*65-67*), similarly shifts C&E
95 onset and suppresses *sumf1* and *sumf2* mutant phenotypes, indicating that heparan sulfate proteoglycans
96 (HSPGs) mediate C&E timing downstream of sulfatase modifiers. Together, these data support a model in
97 which *sumf2* expression at gastrulation onset reduces sulfatase activity, triggering a switch in HSPG sulfation
98 patterns to promote and/or permit C&E morphogenesis.
99
100 **RESULTS**
101
102 ***Ex vivo* convergence & extension requires new transcription at gastrulation onset**
103 Blocking transcription prior to ZGA prevents all gastrulation morphogenesis in zebrafish embryos (*53*).
104 However, it was not determined when new transcription is required for gastrulation cell movements nor the
105 specific genes that are required. We showed previously that otherwise naïve zebrafish embryonic explants
106 expressing Nodal signaling components recapitulate both C&E behaviors and their precise timing in culture
107 while lacking other gastrulation movements (*35*), making them an ideal system to study morphogenesis timing.
108 To identify the temporal window of gene expression required specifically for C&E, we treated explants
109 expressing the constitutively active Nodal receptor Acvr1b* (also known as TARAM-A* (*68*)) with a time-course
110 of the irreversible transcription inhibitor Triptolide (*69*) (**Figure 1A**). *acvr1b** explants treated when intact
111 siblings reached 50% epiboly (5.3 hpf) failed entirely to extend, while those treated shortly thereafter at shield

112 stage (6 hpf, gastrulation onset) or later were able to extend, albeit incompletely under some conditions
113 (**Figure 1B, C**). To rule out the possibility that Triptolide blocks C&E by disrupting Nodal signaling dynamics,
114 we repeated the experiment in explants expressing the Nodal ligand Ndr2/Cyc, which exhibit delayed Nodal
115 activation compared to *acvr1b** explants (*70*), and explants from Nodal signaling-deficient maternal-zygotic
116 (MZ)*oep*-/- embryos (*71*) expressing *acvr1b**, which exhibit continued signaling in the absence of feed-forward
117 synthesis of new Nodal ligands. Both conditions responded identically to wildtype (WT) *acvr1b** explants,
118 indicating that the requirement for transcription is independent of Nodal signaling *per se* (**Figure Suppl. 1A, B,**
119 **D, E**). This is consistent with Activin-induced *Xenopus* animal cap explants extending on time regardless of
120 when Activin was added (*37*). Finally, WT *acvr1b** explants treated with the reversible transcription inhibitor
121 Flavopiridol (*72*) at 50% epiboly and washed out at 90% epiboly (5.3–9 hpf) similarly failed to extend (**Figure**
122 **Suppl. 1C, F**), demonstrating that the stage of inhibition, rather than its duration, was responsible for failed
123 C&E. These results indicate that new gene expression starting at gastrulation onset is required for *ex vivo* C&E
124 movements, independent of Nodal signaling dynamics.
125
126 ***sumf1* and *sumf2* transcript levels invert at gastrulation onset**
127 Having established that transcription at gastrulation onset is crucial for C&E, we next identified genes
128 whose expression is strongly upregulated during this window. We previously profiled transcription in *ndr2,*
129 *acvr1b*,* and uninjected control explants by bulk RNA sequencing at seven developmental stages spanning
130 gastrulation (*70*) (**Figure 1D**). We examined these data for candidate “trigger” genes whose expression
131 exhibited a substantial increase between 50% epiboly and shield stage, corresponding to our experimentally
132 determined requirement for transcription. Because the timing of C&E onset is independent of Nodal signaling
133 dynamics, we only considered genes with similar temporal expression patterns in explants of all three
134 conditions, including uninjected controls. This analysis yielded a list of 180 genes, 129 of which had similar
135 increasing expression patterns in intact zebrafish gastrulae (*73*) (**Table suppl 1**). From these, we selected
136 genes exhibiting at least a 50% increase ($\Delta > 0.5$x) in expression at gastrulation onset (shield stage) from their
137 baseline at 50% epiboly and whose expression at shield stage *in vivo* was over 5 transcripts per million,
138 producing a list of 28 genes (**Figure 1E**) (**Table suppl 1**). Because C&E begins simultaneously in intact

139 embryos and explants, we hypothesized these 28 genes represent candidates whose expression at
140 gastrulation onset may trigger C&E cell behaviors.

141 Among our candidate genes was *sulfatase modifying factor 2* (*sumf2*). *sumf2* encodes pFGE, a paralog
142 and antagonist of Formylglycine Generating Enzyme (FGE) (*64, 74*), encoded by *sulfatase modifying factor 1*
143 (*sumf1*). As its name implies, FGE converts cysteine residues to formylglycine, a rare but essential post-
144 translational modification required within the active site of all sulfatase enzymes for their activity (*74*).
145 Sulfatases catalyze the removal of sulfate groups from a variety of substrates, including lipids, steroids, and
146 glycosaminoglycans, modifying their biological activity (*75, 76*). Both pFGE (*sumf2*) and FGE (*sumf1*) are ER-
147 resident proteins that share high amino acid sequence similarity, with the main distinction that pFGE lacks
148 enzymatic activity (*54, 74*). It is not clear how pFGE (*sumf2*) antagonizes FGE (*sumf1*) function, but evidence
149 suggests that pFGE binds FGE alone or in complex with its sulfatase substrates (*58, 64*). Although other
150 candidates exhibited more dramatic expression increases at gastrulation onset, we selected *sumf2* for further
151 study because its function during vertebrate development has never been explored, and its intriguing
152 complementary temporal expression patterns with *sumf1* during gastrulation. In zebrafish embryos and
153 explants, *sumf1* is maternally expressed but exhibits a sharp reduction in transcript levels just prior to
154 gastrulation onset, coinciding with increased *sumf2 (73)* (**Figure 1F, Figure Suppl. 1G**). Because *sumf2* and
155 *sumf1* encode a negative and positive regulator of sulfatase activity, respectively, we hypothesized that this
156 peak in the *sumf2/sumf1* ratio at gastrulation onset (6hpf) (**Figure 1G**) reduces sulfatase activity and thus
157 increases sulfation of their substrates to trigger C&E movements.

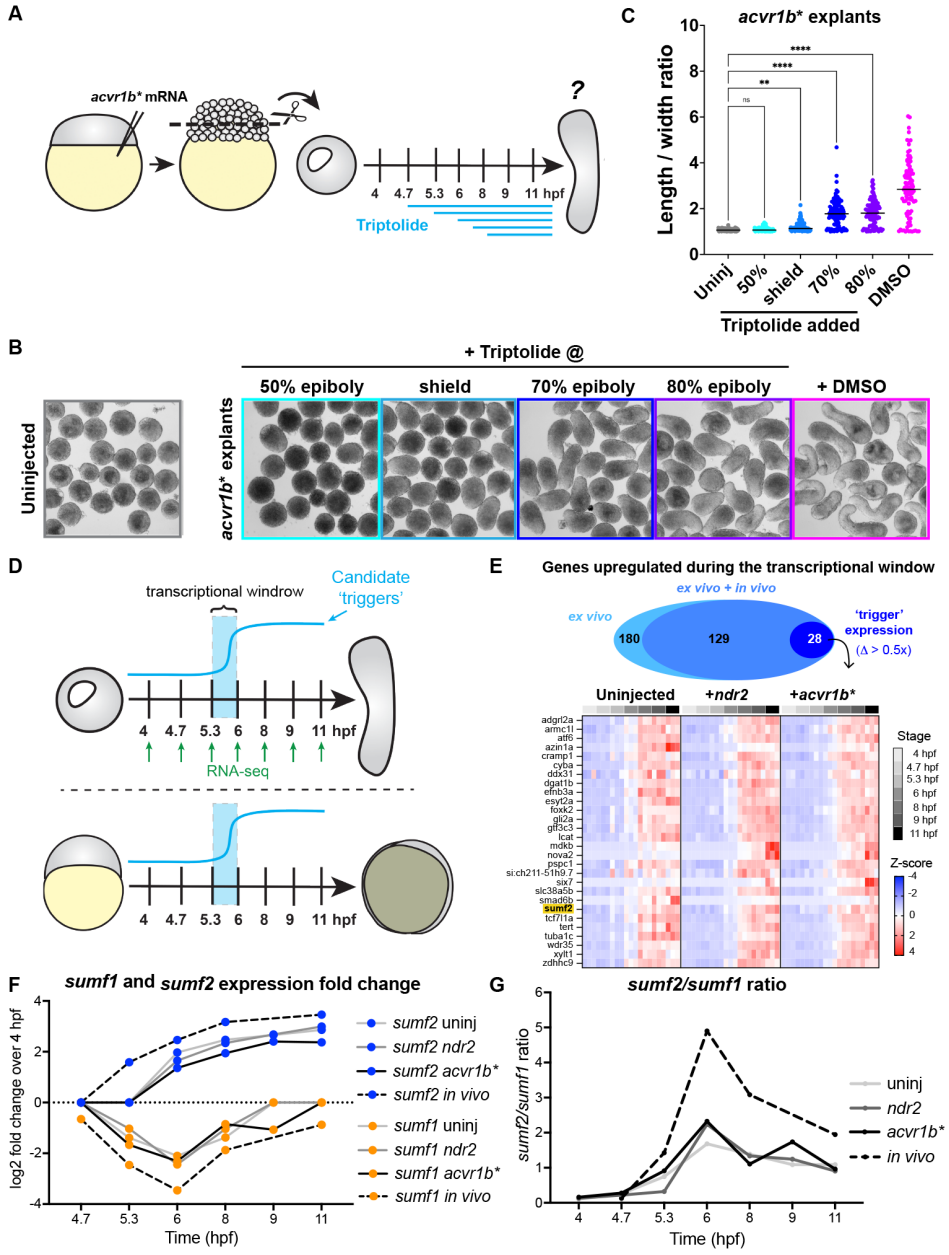

Figure 1. *Ex vivo* convergence & extension requires new transcription at gastrulation onset. (A) Diagram of embryonic explants and Triptolide treatments (modified from (*35*)). **(B)** Representative images of uninjected and *acvr1b** explants at 12 hpf (equivalent of 4-somite stage) after treatment with triptolide at the indicated stages or with DMSO at 50% of epiboly. **(C)** Length/width ratios of explants shown in (B). Each dot represents a single explant from three independent trials, black bars are median values; p<0.0001, Mann-Whitney test. **(D)** Overview of comparisons between published bulk RNA-sequencing experiments from seven developmental stages in three explant conditions (uninjected, *acvr1b**, and *ndr2*) (top) and intact embryos (bottom). Candidate 'trigger' genes were strongly upregulated within the previously determined transcriptional window (5.3–6 hpf). **(E)** (Top) 180 genes exhibited 'trigger' expression patterns in all explant conditions (light blue); of these, 129 were also upregulated in intact embryos (blue), and 28 showed a sharp increase (Δ > 0.5x) with significant expression levels (>5 TPM, dark blue). (Bottom) Heatmap of candidate 'trigger' genes in uninjected, *acvr1b*, and *ndr2* explants. *Sumf2* (highlighted in yellow) was selected for further study. **(F)** Fold-change expression of *sumf1* and *sumf2* transcripts over 4 hpf in explants (solid lines) and embryos (dashed lines) over time. **(G)** *Sumf2/sumf1* expression ratio in explants (solid line) and embryos (dashed line) over time. Note the peak in the *sumf2/sumf1* ratio at 6 hpf, coinciding with the previously determined transcriptional window.

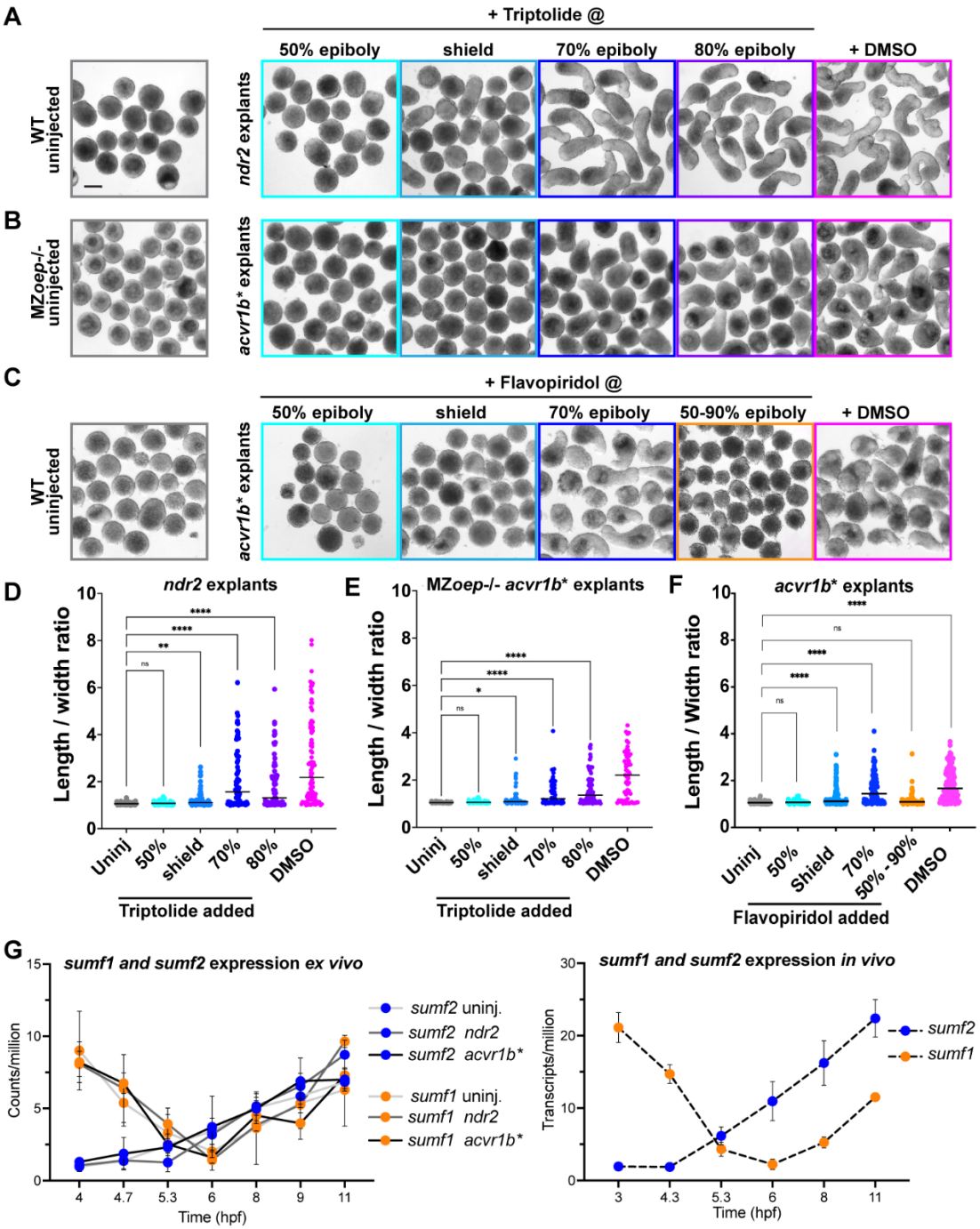

Figure Suppl. 1. *Ex vivo* convergence & extension requires new gene expression at gastrulation onset independently of Nodal signaling dynamics. **(A, B)** Representative images of WT uninjected and *ndr2* explants (A), or MZ*oep*-/- uninjected and *acvr1b** explants (B) at 12 hpf (equivalent of 4-somite stage) after treatment with triptolide at the indicated stages or with DMSO at 50% of epiboly. Scale bar 200 μm. **(C)** Representative images of WT uninjected and *acvr1b** explants at 12 hpf (equivalent of 4-somite stage) after treatment with reversible transcription inhibitor Flavopiridol at the indicated stages or with DMSO at 50% of epiboly. **(D-F)** Length/width ratios of explants shown in (A, B and C), respectively. Each dot represents a single explant from three independent trials, black bars are median values; $p<0.0001$, Mann-Whitney test. **(G)** Expression levels of *sumf1* and *sumf2* in uninjected (light grey lines), *ndr2* (grey lines) and *acvr1b** (black lines) explants (left), and embryos (dashed lines, right) over time. Dots represent the mean of three (for explants) or five (for embryos) bulk RNA-seq replicates and error bars represent standard deviation.

188 **Excess or loss of sulfatase modifying factors causes C&E defects in zebrafish gastrulae**

189 To investigate the role of sulfatase modifying factors in C&E morphogenesis, we employed both gain- and
190 loss-of-function approaches and performed morphometric analysis at the end of gastrulation (tailbud stage, 10
191 hpf). We first overexpressed *sumf1* or *sumf2* by mRNA injection into single-cell WT embryos. *sumf1*
192 overexpressing (OE) gastrulae exhibited a significant reduction in anteroposterior (AP) axis length and wider
193 notochords, characteristic of C&E defects, and *sumf2* OE embryos showed similar but milder phenotypes
194 (**Figure 2**). We next examined gastrulae of the recently characterized *sumf1*^{la015919Tg} mutant line (hereafter
195 *sumf1*-/-), which harbors a viral insertion in exon 1 and abolishes FGE activity (*77*). Homozygous mutant
196 embryos survived to adulthood (*77*), enabling examination of MZ*sumf1*-/- gastrulae, which also exhibited
197 reduced AP axis length and wider notochords (**Figure 2**). Because no existing *sumf2* mutant line was
198 available, we used CRISPR to generate a 7 bp insertion in exon 2 of *sumf2* (named *bcm126*), resulting in a
199 premature stop codon (**Figure Suppl. 2A**). Homozygous *sumf2*$^{bcm126/bcm126}$ embryos (hereafter *sumf2*-/-),
200 showed a substantial reduction in *sumf2* transcript levels by qRT-PCR (**Figure Suppl. 2C**), confirming this as a
201 strong loss-of-function allele. These mutants also survived to adulthood, enabling us to maintain the line as
202 homozygotes and analyze MZ*sumf2*-/- embryos. Notably, approximately 10% of homozygous larvae displayed
203 a tail-curled-up phenotype at 48 hpf and a variable number of *sumf2*-/- adults were undersized and scoliotic
204 (**Figure Suppl. 2D, E**). Although MZ*sumf2*-/- mutant gastrulae did not exhibit C&E defects, *sumf1* OE in this
205 background produced more severe C&E phenotypes than in WT embryos (**Figure 2**), supporting an
206 antagonistic role for *sumf2*/pFGE on *sumf1*/FGE. Together, these results demonstrate that altering levels of
207 *sumf1* or/and *sumf2* disrupts C&E gastrulation morphogenesis *in vivo*.

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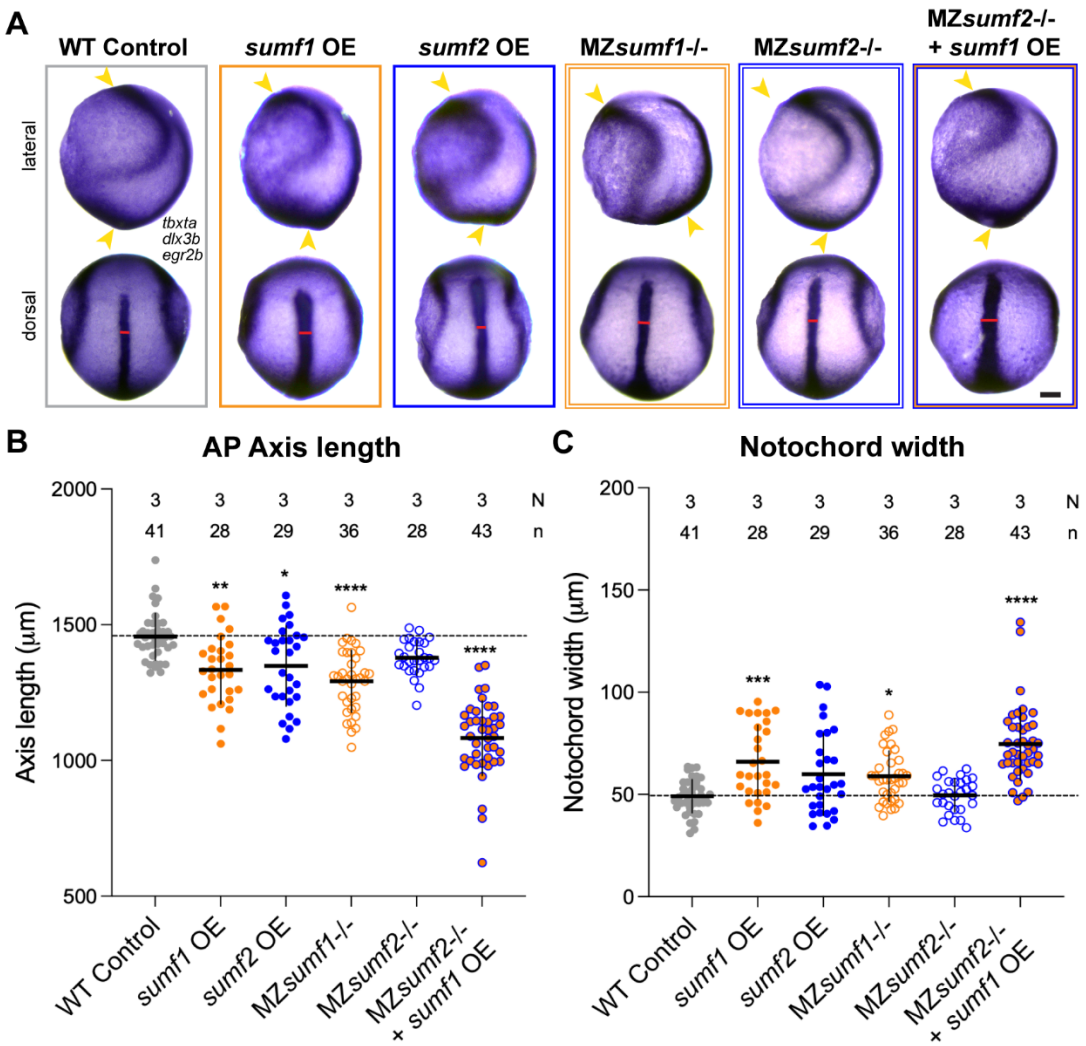

Figure 2. C&E defects in zebrafish gastrulae with altered sulfatases modifiers factor levels. **(A)** Representative images of whole mount in situ hybridization (WISH) for *tbxta* (mesoderm), *dlx3b* (neural plate border) and *egr2b* (rhombomeres 3 & 5) in tailbud stage (10 hpf) embryos of the indicated conditions. Anterior is up in all images, lateral views are shown on top, dorsal views on bottom. Scale bar 100 µm. **(B, C)** Anteroposterior (AP) axis length (B, yellow arrowheads) and notochord width (C, red lines) of embryos depicted in (A). Each dot represents a single embryo, N: number of independent experiments, n: number of embryos. Means and standard deviation are indicated; * $p<0.05$, ** $p<0.01$, **** $p<0.0001$ compared to WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests.

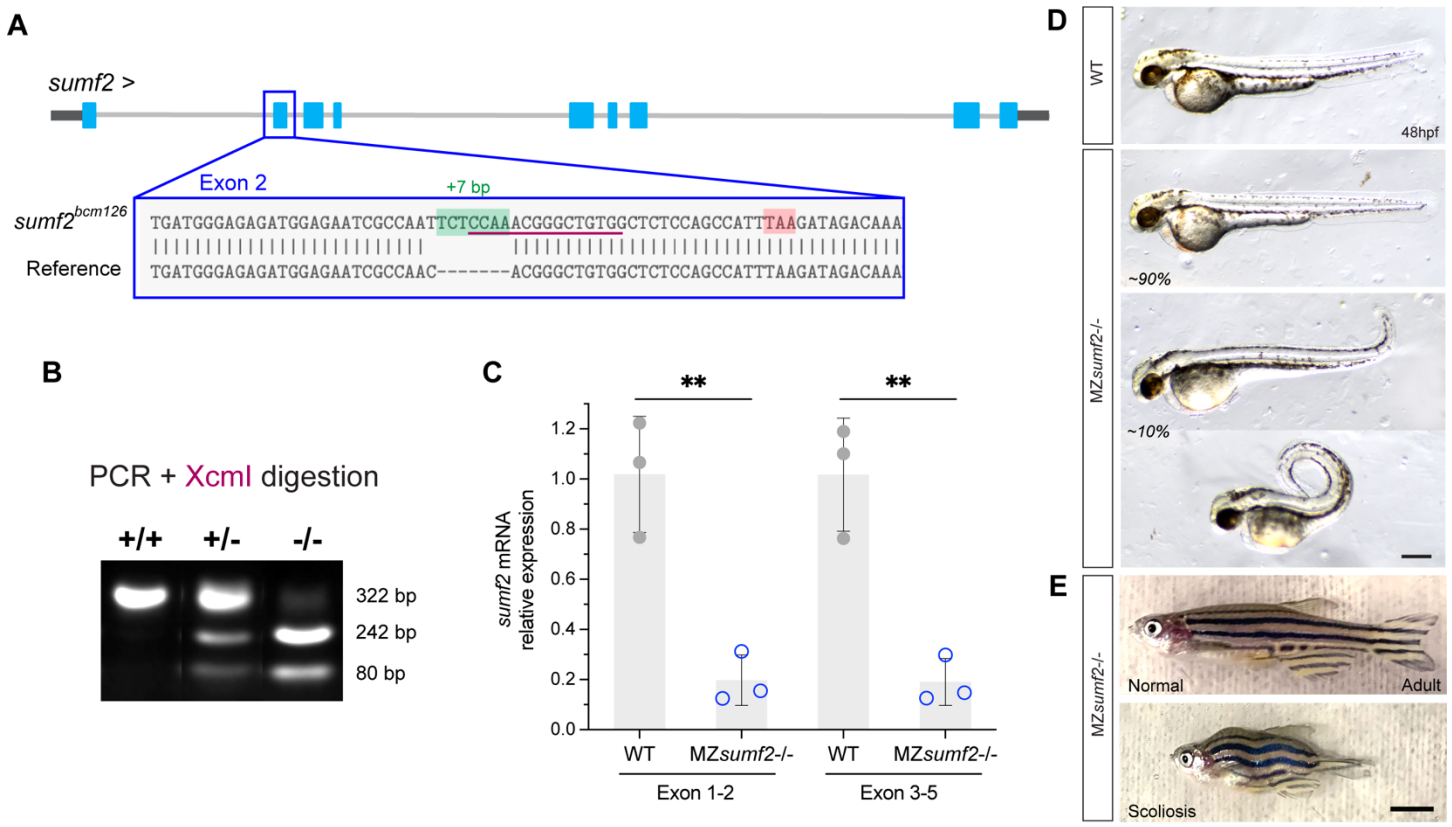

Figure Suppl. 2 Characterization and genotyping of new *sumf2* loss-of-function allele. (A) Diagram of CRISPR-induced 7 bp insertion (green) in exon 2 of *sumf2* (*sumf2*^{bcm126}), resulting in a premature stop codon (red). **(B)** The *sumf2*^{bcm126} allele creates an XcmI restriction site (purple underline in (A)), enabling genotyping by digestion of a 322 bp PCR-amplified fragment, which yields 242 bp and 80 bp products in heterozygous (+/−), and homozygous (−/−) but not WT (+/+) samples. **(C)** Relative expression of *sumf2* transcripts in WT and MZ*sumf2* -/- larvae, measured by RT-qPCR using primers spanning *sumf2* exon 1–2 and exon 3–5. Means and standard deviation are indicated. Each dot represents one of three independent clutches; ** $p<0.01$, Mann-Whitney test. **(D)** Representative images of WT and MZ*sumf2* -/- larvae at 48 hpf. Approximately 90% of MZ*sumf2* -/- larvae developed normally, while ~10% displayed varying degrees of tail-curled-up phenotypes. Anterior is to the left; scale bar 200 μm. **(E)** Representative images of MZ*sumf2* -/- adult fish showing *Normal* and *Scoliosis* phenotypes. Scale bar 500 μm.

232 ***sumf1* and *sum2* levels modify the timing of C&E *ex vivo***

233 We next examined the effects of altered *sumf1* and *sumf2* levels in zebrafish embryonic explants, in which
234 C&E is isolated from other concurrent morphogenetic processes. Surprisingly, neither OE nor deficiency of
235 *sumf1* or *sumf2* impaired the extension of *acvr1b** explants at the equivalent of 4-somite stage (**Figure Suppl.**
236 **3**), indicating that they are dispensable for C&E *per se*. To assess whether *sumf1* or *sumf2* levels instead
237 influenced the timing of C&E morphogenesis, we acquired time-lapse recordings of *acvr1b** explants from
238 which we determined the precise onset of morphological extension (**Figure 3A**). Importantly, all explants were
239 staged relative to blastopore closure in age-matched intact embryos of the same genetic background mounted
240 in the same dish. As described previously (*35*), WT *acvr1b** explants (WT control) and controls co-injected with
241 *superfold-GFP* mRNA (*sf-GFP* OE) initiated extension around 8hpf. Strikingly, extension began significantly
242 later upon *sumf1* OE and significantly earlier upon *sumf2* OE (**Figure 3B**). Co-overexpression of *sumf1* and
243 *sumf2* restored the normal time of extension onset (**Figure 3B**), demonstrating that the balance between these
244 factors (rather than their absolute levels) determines the time of C&E onset. In the converse loss-of-function
245 experiments, we found that extension onset was precocious in MZ*sumf1*-/- explants and delayed in MZ*sumf2*-/-
246 explants (**Figure 3A, B**), consistent with - but opposite to - *sumf1* and *sumf2* OE phenotypes. MZ*sumf1*-/-,
247 MZ*sumf2*-/- double mutant explants phenocopied the precocious extension of MZ*sumf1*-/- single mutants,
248 consistent with *sumf2/*pFGE modulating C&E timing via its role as an antagonist of *sumf1*/FGE. Together,
249 these results demonstrate that the ratio of *sumf1/sumf2* governs the timing of explant extension, with higher
250 *sumf1* delaying and higher *sumf2* advancing C&E onset.

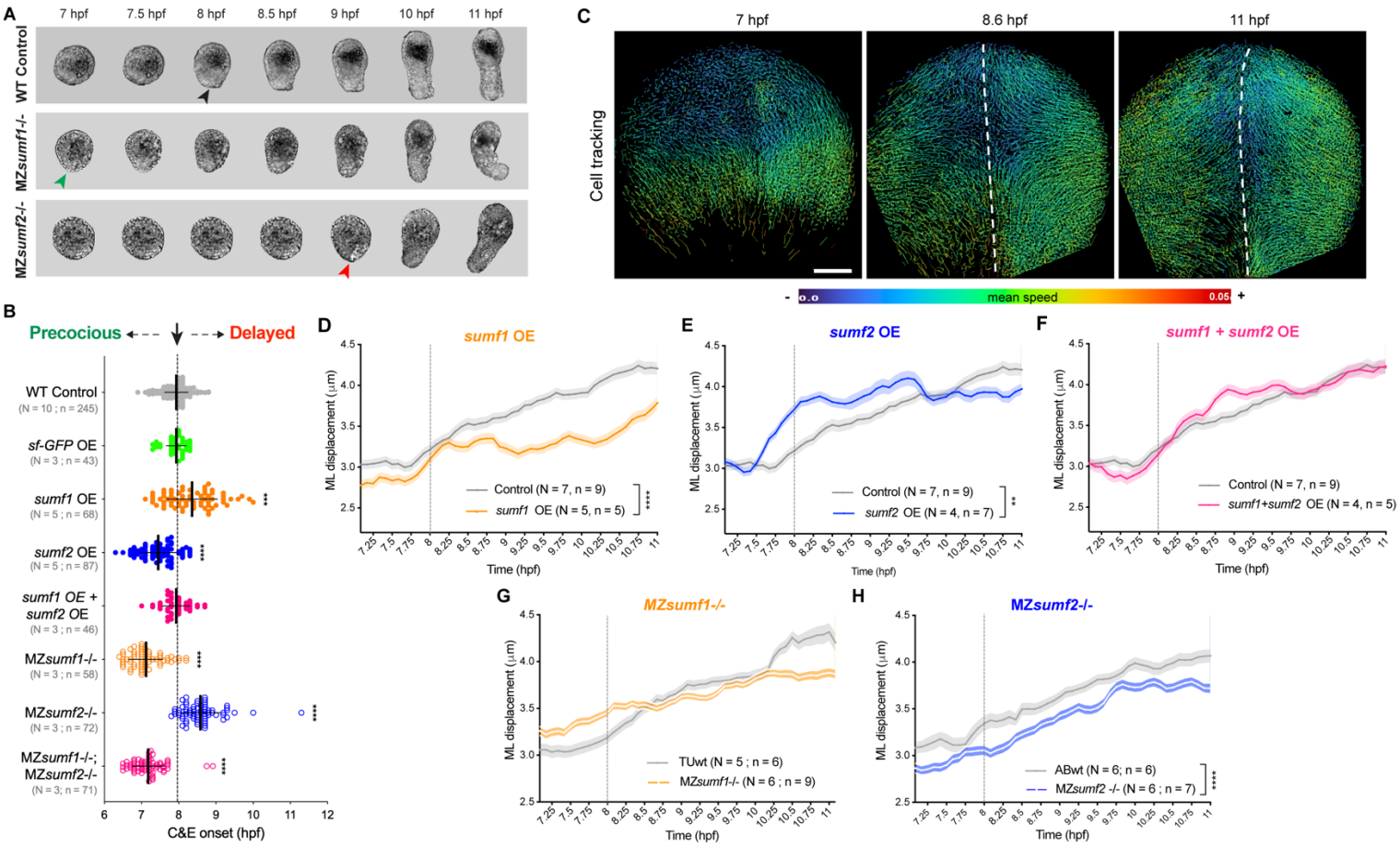

Figure 3. *sumf1* and *sum2* levels control the timing of C&E. (A) Representative bright-field images of *acvr1b** explants of the indicated genotype over time. Black, red, and green arrowheads indicate timely, precocious, and delayed onset of extension, respectively. **(B)** Onset of extension in *acvr1b** explants of the indicated conditions. Dotted line shows typical extension onset of WT control explants around 8 hpf. Each dot represents a single explant. Means and standard deviation are indicated, N: number of independent experiments, n: number of explants, *** p<0.001, **** p<0.0001 compared with WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests. **(C)** Representative images of automated nuclear tracking in the dorsal hemisphere of zebrafish gastrulae, starting before C&E onset (7 hpf). Tracks are color-coded by mean speed; dashed lines mark the dorsal midline; scale bar 100 μm. **(D-H)** ML cell displacement over time for WT control (gray lines) and *sumf1* OE (D), *sumf2* OE (E), *sumf1* + *sumf2* OE (F), MZ*sumf1*-/- (G), and MZ*sumf2*-/- (H) embryos. Dotted line shows typical onset of convergence movements in WT control embryos around 8 hpf. Means and standard error are indicated, N: number of independent experiments, n: number of embryos. ** p<0.01, **** p<0.0001 compared with WT control group by Wilcoxon signed-rank test.

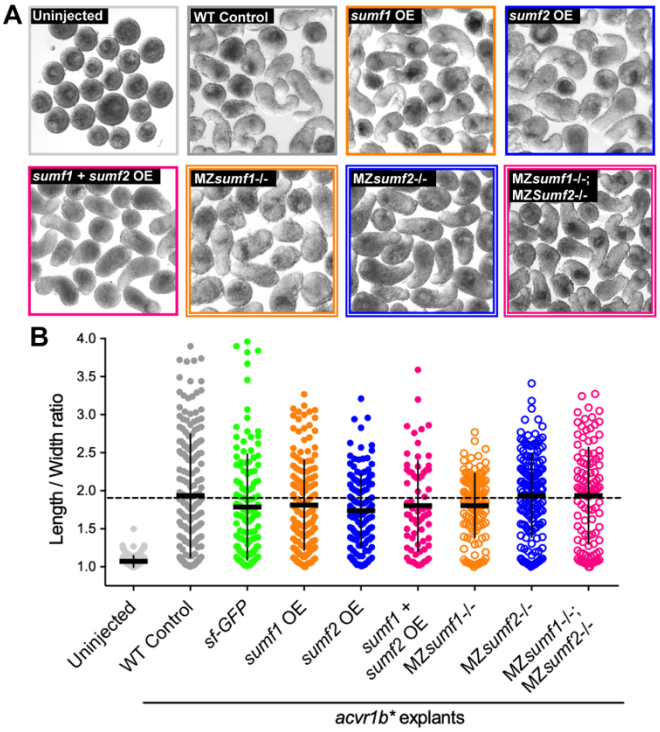

Figure Suppl. 3 Altered *sumf1* and *sumf2* levels do not impair extension of Nodal-induced explants (A) Representative images of uninjected and *acvr1b** explants from the indicated experimental conditions. **(B)** Length/width ratios of explants at the equivalent of 4-somite stage (12 hpf) shown in (A). Each dot represents a single explant from three independent trials. Means and standard deviation are indicated. p>0.05, Kruskal–Wallis test.

276 ***sumf1* and *sum2* levels modify the timing of C&E *in vivo***

277 To determine whether *sumf1* and *sumf2* similarly modify the timing of C&E cell movements *in vivo*, we
278 acquired confocal time-lapse movies of *H2B-scarlet*-labeled gastrulae from 6.5 – 11 hpf and performed
279 automated nuclear tracking (see *Methods*; **Figure 3C**). To ensure accurate stage matching across
280 experiments, all embryos were staged relative to formation of the second somite, which was readily visible at
281 the end of each movie. Convergence movements were quantified as the mediolateral (ML) displacement of
282 dorsal and lateral cells over time (**Figure 3D–H**). Because convergence movements are reduced within the
283 embryonic midline where extension is more prominent (*78*), cells within 100 μm of the dorsal midline were
284 excluded from our analysis. In WT control embryos, we observed an upward inflection of ML displacement
285 beginning around 7.75 - 8 hpf, marking the onset of convergence movements (consistent with previous findings
286 (*18, 19*)), which continued to increase gradually thereafter (**Figure 3D–H**). Upon *sumf1* OE, baseline
287 convergence speed was reduced and, although convergence movements began at the same time as controls,
288 they remained substantially slower (**Figure 3D)**. *sumf2* OE embryos, on the other hand, had similar baseline
289 convergence speeds as controls but with an earlier inflection point (**Figure 3E)**. For much of gastrulation, these
290 movements were also faster than those of controls, indicating a likely change in both onset and pace of
291 convergence movements. As in explants, co-OE of both *sumf1* and *sumf2* restored convergence movements to
292 control timing and speeds (**Figure 3F)**. Also as in explants, *in vivo* cell tracking of MZ*sumf1-/-* and MZ*sumf2-/-*
293 mutants revealed opposite effects to their overexpression. Convergence onset in MZ*sumf1-/-* embryos trended
294 earlier than controls and baseline speed trended higher, although these movements plateaued later in
295 gastrulation (**Figure 3G)**. Conversely, baseline convergence in MZ*sumf2-/-* mutants was slower than controls
296 with a later inflection point, indicating delayed convergence (**Figure 3H)**. Together with our *ex vivo* results,
297 these data support a model in which *sumf2*/pFGE counters the activity of *sumf1*/FGE to control the onset of
298 C&E cell movements during gastrulation.

299

300 **Sulfatase modifiers govern C&E via the extracellular sulfatase Sulf1**

301 Because *sumf1*/FGE and *sumf2*/pFGE together regulate sulfatase activity levels, we hypothesized that
302 high *sumf2/sumf1* ratios trigger C&E onset by reducing the activity of one or more key sulfatases. Indeed,
303 increased sulfatase activity was shown to disrupt gastrulation morphogenesis in *Xenopus* and sea urchin (*63,*
304 *79-82*). The zebrafish genome encodes 17 sulfatases, 11 of which are expressed during peri-gastrulation
305 stages (*73*) (**Figure Suppl. 4A**). To identify which of these mediates the effect of *sumf1/sumf2* on C&E timing,
306 we overexpressed each of these 11 sulfatases and performed morphometric analysis at tailbud stage and
307 scored axis phenotypes at 24 hpf. Notably, three of them — the heparan sulfate endosulfatases *sulf1* and
308 *sulf2a*, and the chondroitin sulfatase *arsb* — produced severe AP axis extension defects and increased
309 notochord width, consistent with C&E defects (**Figure Suppl. 4B-E**). Unlike *sumf1* and *sumf2* OE,
310 overexpression of these three sulfatases also prevented full extension of *acvr1b** explants at the equivalent of
311 4-somite stage (**Figure Suppl. 4F, G**). However, only *sulf1* OE significantly delayed *acvr1b** explant extension,
312 while the other two had no effect on C&E timing (**Figure 4A**). Thus, of the 17 zebrafish sulfatases, only one
313 was found to affect both C&E morphogenesis and its timing.

314 To further investigate the role of Sulf1 in zebrafish gastrulation morphogenesis, we examined C&E in
315 MZ*sulf1*$^{sjr9/sjr9}$ full-locus deletion mutants (Kaur Bajwa et al, in submission) (hereafter MZ*sulf1-/-*). MZ*sulf1-/-*
316 embryos exhibited reduced AP axis length at tailbud stage (**Figure 4B-D)** and MZ*sulf1-/- acvr1b** explants
317 showed impaired extension (**Figure Suppl. 4F-G)**, indicating that both gain and loss of Sulf1 reduces C&E.
318 Strikingly, MZ*sulf1-/- acvr1b** explants exhibited precocious extension (**Figure 4A**), similar to MZ*sumf1-/-* and
319 *sumf2* OE explants (but opposite to *sulf1* OE). We next quantified the effect of Sulf1 levels on C&E onset *in*
320 *vivo*. Convergence speed was drastically reduced upon *sulf1* OE (**Figure 4E**) while MZ*sulf1-/-* gastrulae
321 exhibited earlier and enhanced convergence movements compared to WT controls (**Figure 4F**). These results
322 highlight Sulf1 as a strong candidate for the key sulfatase controlling C&E onset.

323 To examine the relationship between Sulf1 and *sumf1*/FGE, we generated *acvr1b** explants co-injected
324 with low doses of each mRNA alone or in combination. The combination of otherwise sub-phenotypic doses of
325 *sumf1* and *sulf1* synergistically delayed and reduced explant extension (**Figure 4G, Figure Suppl. 4F, G),**
326 consistent with *sumf1*/FGE enhancing Sulf1 activity. Finally, we tested whether *sumf1* OE could delay explant
327 extension in the absence of *sulf1*. We found that although *sumf1* OE significantly delayed extension onset in

WT explants, it had no effect on the onset of extension in MZ*sulf1*-/- explants, which remained precocious (**Figure 4G**). Together, these results implicate Sulf1 as the primary sulfatase through which *sumf1*/FGE and *sumf2*/pFGE govern the timing of C&E morphogenesis.

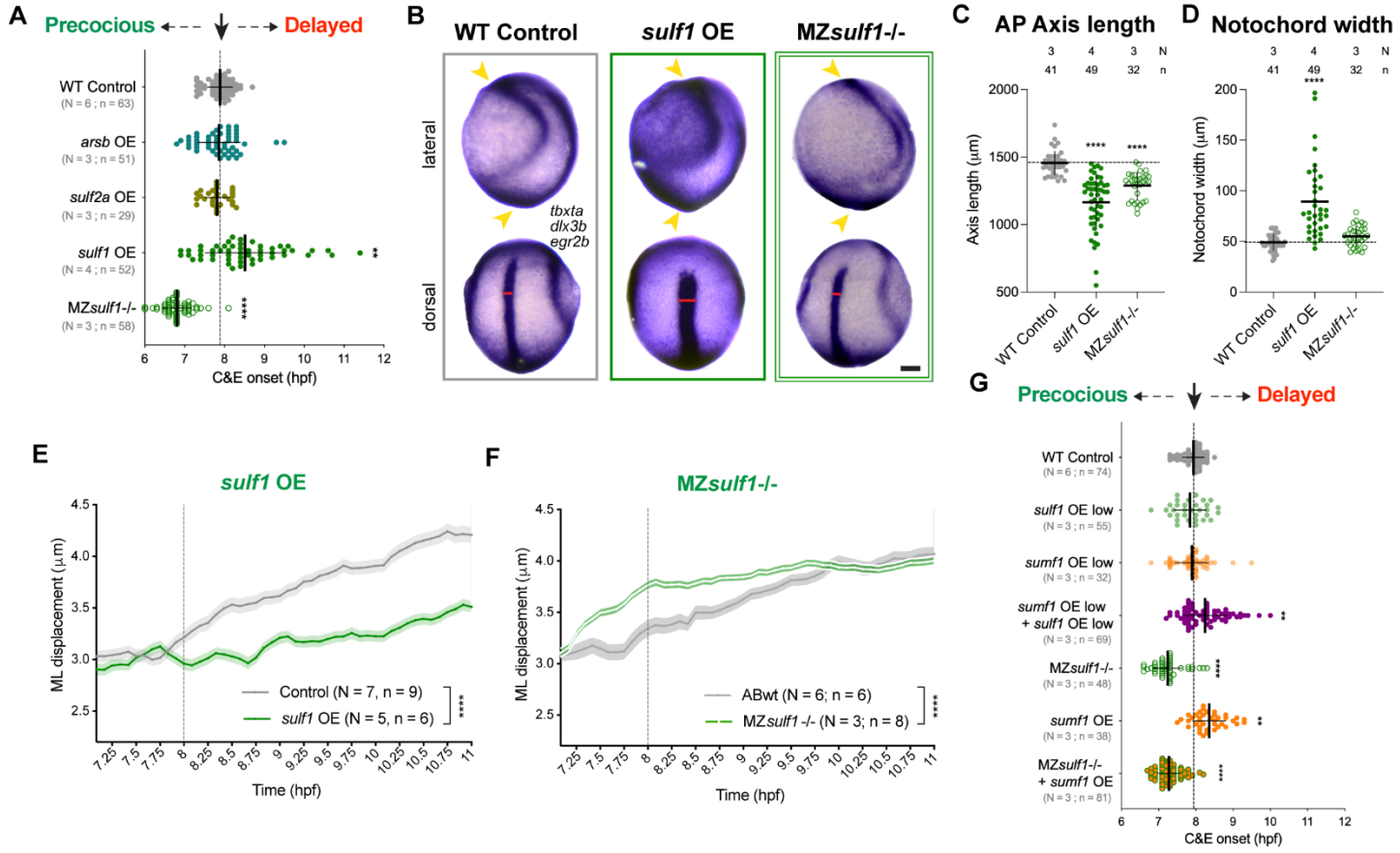

Figure 4. The extracellular sulfatase Sulf1 governs C&E and its timing. (A) Onset of extension in *acvr1b** explants of the indicated conditions. Dotted line shows typical extension onset of WT control explants around 8 hpf. Each dot represents a single explant. Means and standard deviation are indicated, N: number of independent experiments, n: number of explants. *** p<0.001, **** p<0.0001 compared with WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests. **(B)** Representative images of WISH for *tbxta* (mesoderm), *dlx3b* (neural plate border) and *egr2b* (rhombomeres 3 & 5) in tailbud stage (10 hpf) embryos of the indicated conditions. Anterior is up in all images, lateral views are on top, dorsal views on bottom. Scale bar 100 µm. **(C, D)** Anteroposterior (AP) axis length (C) and Notochord width (D) were quantified as in Figure 2 from embryos depicted in (B). Each dot represents a single embryo. Means and standard deviation are indicated. **** p<0.0001 compared with WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests. **(E, F)** ML cell displacement from automated nuclear tracking (as in Figure 3) in WT control and *sulf1* OE (E) and MZ*sulf1*-/- (F) zebrafish gastrulae. Means and standard error are indicated. N: number of independent experiments, n: number of embryos. **** p<0.0001 compared with WT control group by Wilcoxon signed-rank test. **(G)** Onset of extension in *acvr1b** explants of the indicated conditions. Dotted line shows typical extension onset of WT control explants around 8 hpf. Each dot represents a single explant. Means and standard deviation are indicated, N: number of independent experiments, n: number of explants. ** p<0.01, **** p<0.0001 compared with WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests.

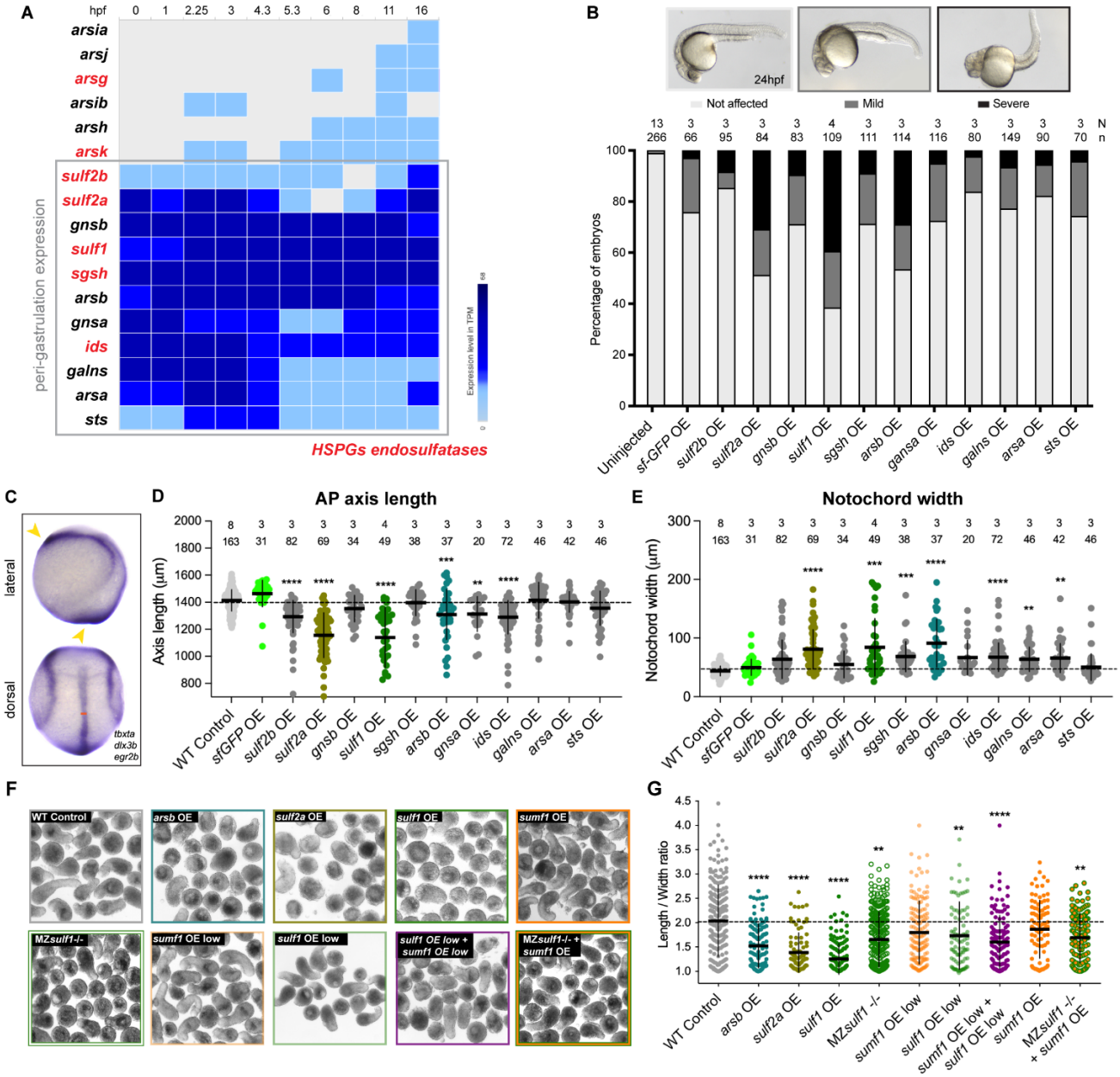

Figure Suppl. 4. Evaluation of C&E defects upon sulfatase overexpression. (A) Expression levels of all 17 zebrafish sulfatases over time based on publicly available bulk RNA-seq data. Gray square indicates the 11 expressed during peri-gastrulation, HSPG endosulfatases are in red. **(B)** (Top) Representative images of 24 hpf embryos overexpressing each of the 11 peri-gastrulation sulfatases displaying a range of axis phenotypes scored as *"Not affected"*, *"Mild"* or *"Severe"*. (Bottom) Mean percentage of 24 hpf embryos of the conditions indicated exhibiting each class of axis defect. N: number of independent experiments, n: number of embryos. **(C)** WISH for *tbxta* (mesoderm), *dlx3b* (neural border) and *egr2b* (rhombomeres 3 & 5) in tailbud stage (10 hpf) embryos shown from lateral (top) and dorsal (bottom) views. **(D, E)** Anteroposterior (AP) axis length (D) and Notochord width (E) (as in Figure 2) in control and sulfatase-overexpressing embryos. Each dot represents a single embryo. Means and standard deviation are indicated. (** p<0.01, *** p<0.001, **** p<0.0001 as compared with WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests. **(F)** Representative images of *acvr1b** explants from the indicated experimental conditions. **(G)** Length/width ratios of 12 hpf explants shown in (F). Each dot represents a single explant. Means and standard deviation are indicated. * p>0.05, ** p<0.01, **** p<0.0001 compared with WT control group by Kruskal–Wallis and Dunn's multiple comparisons tests.

368 **Sulfated heparan sulfate proteoglycans increase with C&E onset**

369 Sulf1 is an extracellular sulfatase that removes 6-*O* sulfation from GlcA-GlcNS6S (D0S6) and IdoA2S-
370 GlcNS6S (D2S6) disaccharide units of heparan sulfate proteoglycans (HSPGs)(*66*). We would therefore
371 expect the inversion of *sumf1*/FGE and *sumf2*/pFGE levels at the beginning of gastrulation to decrease Sulf1
372 activity, leading to increased sulfated HSPG levels at late gastrulation. Indeed, Alcian Blue staining (at pH ~1)
373 (*83*) for overall glycosaminoglycan (GAG) sulfation increased dramatically from early (50% epiboly) to late
374 (90% epiboly) gastrulation stages, indicating increased embryo-wide GAG sulfation. Treatment with sodium
375 chlorate, which prevents the formation of the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS)
376 (*84, 85*), drastically diminished Alcian Blue staining (**Figure 5A**), confirming its specificity.

377 Because Alcian Blue stains all negatively charged GAGs, we next quantified levels of sulfated heparan
378 sulfate (HS) disaccharides by hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-
379 LC/MS) in early and late zebrafish gastrulae (*86*). We found that total HS levels increased significantly between
380 50% and 80% epiboly stages (**Figure 5B**). Although we observed no changes in the percentage of the 6-*O*
381 sulfated D0S6 and D2S6 disaccharides between stages (**Figure 5C**), we did find a significant increase in total
382 levels of these 6-*O* sulfated disaccharides at late gastrulation (reflecting the observed increase in total HS
383 levels) (**Figure 5D**). These results indicate that sulfation of Sulf1 substrates increases during gastrulation,
384 consistent with our hypothesis that the peak *sumf2/sumf1* ratio at gastrulation onset leads to reduced Sulf1
385 activity.

386

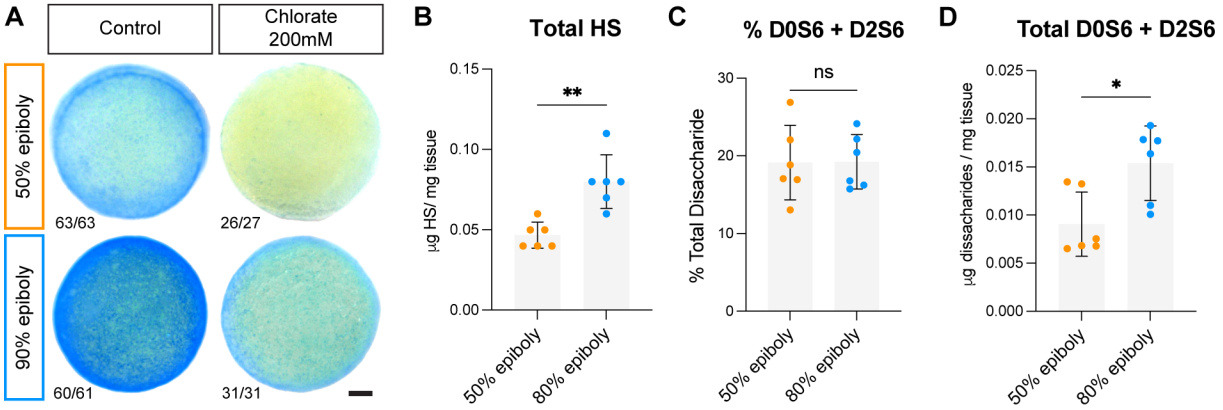

Figure 5. Heparan sulfate proteoglycans and their sulfation increase during gastrulation. (A) Representative images of zebrafish early (50% epiboly, 5.3 hpf) and late (90% epiboly, 9 hpf) gastrulae stained with Alcian Blue for sulfated GAGs. Alcian Blue staining was diminished in zebrafish embryos treated at dome stage (4.3 hpf) with 200 mM sodium chlorate. Fractions indicate the number of embryos with the depicted staining pattern over number of embryos examined. Scale bar 100 µm. **(B-D)** HILIC-LC/MS analysis of early and late zebrafish gastrulae showing the total amount of Heparan Sulfate (HS) (B), the percentage of the D0S6 and D2S6 disaccharides (C), and the total amount of D0S6 and D2S6 disaccharides (D) at the stages indicated. Plots show mean and standard deviation, with each dot representing an independent clutch of 50 embryos, ** $p<0.01$, * $p< 0.05$, Mann-Whitney test.

398 **HSPGs mediate the effects of sulfatase modifiers on C&E timing**

399 Sulfatases modify a large number of biological substrates, including lipids, steroids, and multiple GAGs
400 (*87*). To test whether sulfated HSPGs influence C&E morphogenesis and its timing, we experimentally
401 decreased and increased sulfated HS levels within embryos and explants. To inhibit overall sulfation, including
402 GAGs, we treated dome stage embryos (4.3 hpf) with two different doses of sodium chlorate (200 mM and 50
403 mM), which we previously showed reduced Alcian Blue staining in zebrafish gastrulae (**Figure 5A**). We
404 observed severe AP axis shortening and cyclopia in 24 hpf embryos treated with the higher dose, and milder
405 axis defects at the lower dose (**Figure 6A**). A dose-dependent effect on C&E was also observed at tailbud
406 stage (**Figure 6B-D**), demonstrating that sulfation is required for proper C&E morphogenesis (as previously
407 reported (*88-90*)). Although explants treated with the higher dose were not viable, treatment with the lower
408 dose of sodium chlorate also dramatically reduced extension of *acvr1b∗* explants (**Figure Suppl 5A, B**).
409 Sodium chlorate treatment also significantly delayed extension onset in both WT control and, importantly, in
410 MZ*sumf1*-/- explants in which extension otherwise occurred early (**Figure 6E**, **Figure Suppl 5C**). This finding
411 strongly suggests that loss of *sumf1* induces precocious C&E by enhancing sulfation.

412 In a complementary experiment, we increased levels of sulfated HS by injecting the extracellular space of
413 256-cell stage embryos with 10 ng/ml HS (or water as a control) (**Figure 6F**). As previously reported (*91*), HS-
414 injected embryos exhibited dorsalized phenotypes (**Figure Suppl. 5D**), likely reflecting increased FGF
415 signaling (*92*). Notably, while increased HS did not affect the extension of *acvr1b∗* explants at the equivalent of
416 4-somite stage (**Figure Suppl. 5A, B**), their extension onset was significantly earlier than control explants
417 (**Figure 6G, Figure Suppl 5E**). HS injection also advanced extension onset in MZ*sumf2*-/- explants (**Figure
418 6G, Figure Suppl 5E**), indicating that increased HS levels are sufficient to trigger precocious C&E *ex vivo* and
419 can override the delay caused by *sumf2* deficiency. Taken together, these results support a model in which
420 *sumf2* expression at gastrulation triggers the timely onset of C&E cell movements by reducing Sulf1 activity
421 and consequently increasing sulfation of HSPGs.

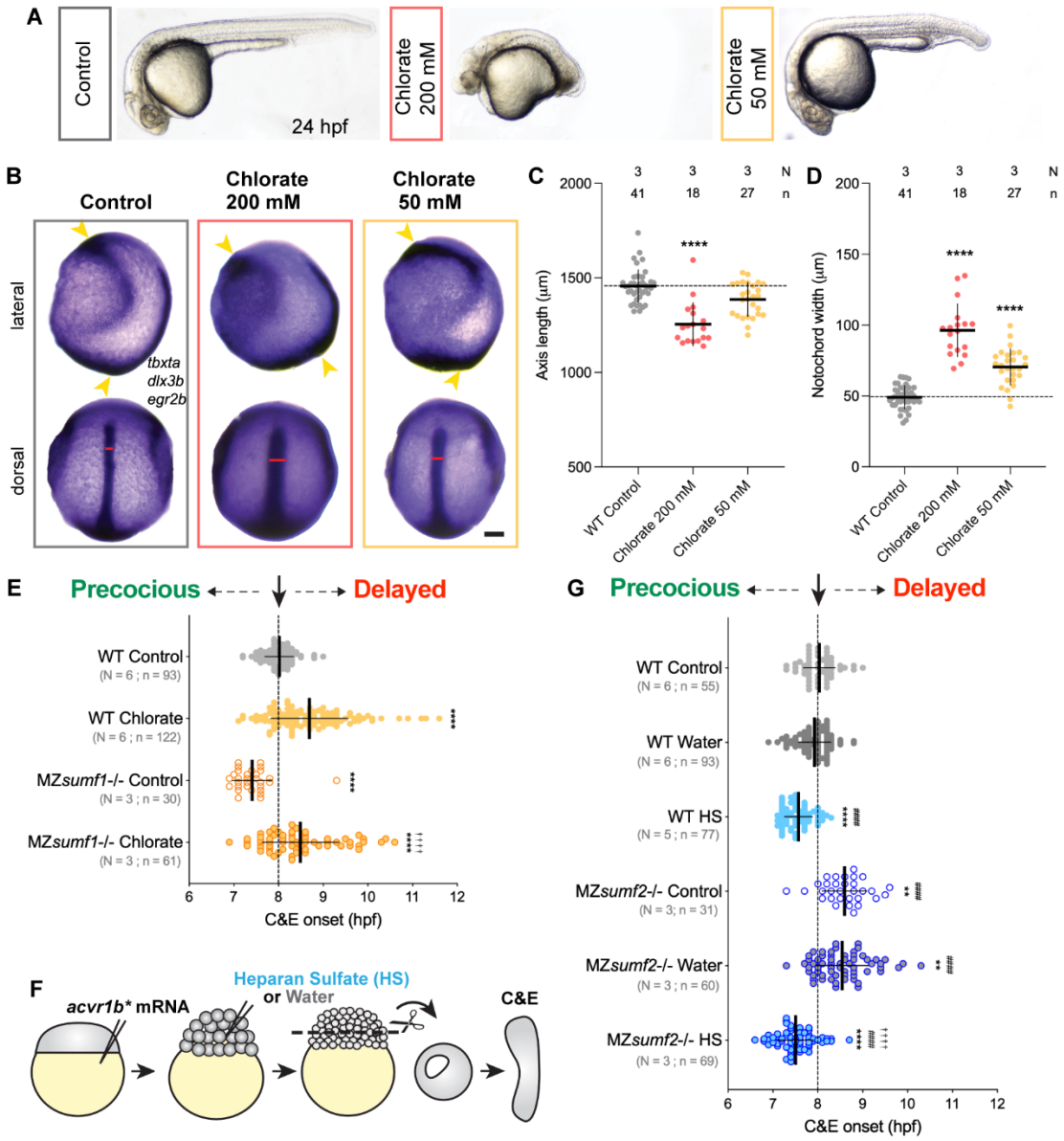

Figure 6. HSPGs mediate the effect of sulfatase modifying factors on C&E timing. (A) Representative images of 24 hpf zebrafish embryos treated at dome stage (4.33 hpf) with 200 mM or 50 mM of sodium chlorate. **(B)** Representative images of WISH for *tbxta* (mesoderm), *dlx3b* (neural border) and *egr2b* (rhombomeres 3 & 5) in tailbud stage (10 hpf) control and sodium chlorate treated embryos shown from lateral (top) and dorsal (bottom) views. Scale bar 100 µm. **(C, D)** AP axis length (C) and Notochord width (D) (as in Figure 2) of embryos depicted in (B). Each dot represents a single embryo. Means and standard deviation are indicated. **** p<0.0001 compared with WT control embryos by Kruskal–Wallis and Dunn's multiple comparisons tests. **(E)** Onset of extension in *acvr1b** explants of the indicated conditions. Dotted line shows typical extension onset of WT control explants around 8 hpf. Each dot represents a single explant. Means and standard deviation are indicated. N: number of independent experiments, n: number of embryos. ****/ †††† p<0.0001 compared with WT and MZ*sumf1*-/- control groups, respectively, by Kruskal–Wallis and Dunn's multiple comparisons tests. **(F)** Diagram of extracellular heparan sulfate (HS) or water (control) injections in 256-cell embryos prior to explantation (modified from (*35*)). **(G)** Explant extension onset as in (E). ** p>0.01; ****/ ####/ †††† p<0.0001 compared with WT, WT + water, and MZ*sumf2*-/- + water groups, respectively, by Kruskal–Wallis and Dunn's multiple comparisons tests.

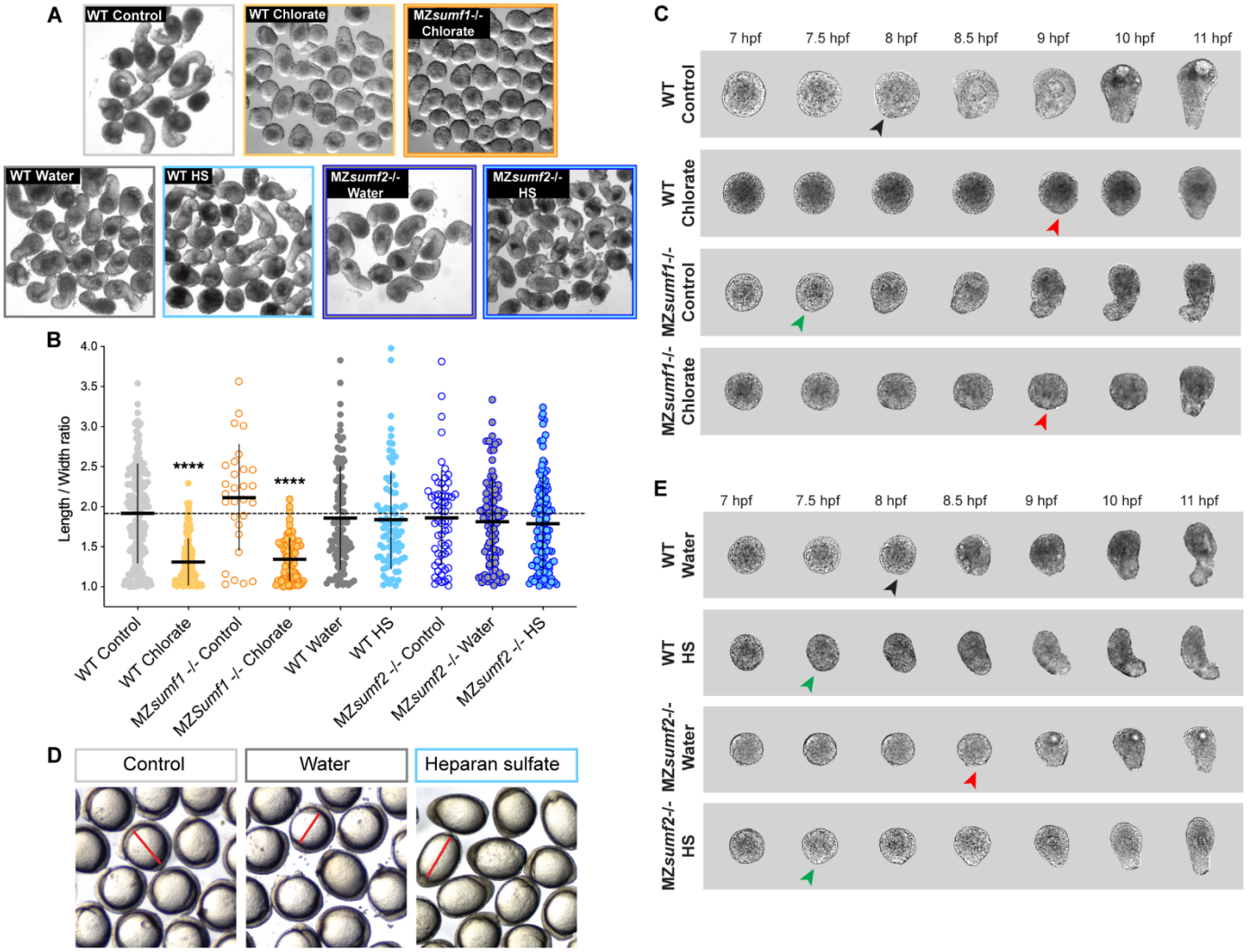

Figure Suppl. 5. Inhibition of sulfation, but not increased heparan sulfate levels, impairs C&E *ex vivo*. **(A)** Representative images of *acvr1b** explants from the indicated conditions. **(B)** Length/width ratios of explants shown in (A). Each dot represents a single explant. Means and standard deviation are indicated. **** $p<0.0001$ compared to WT controls by Kruskal–Wallis and Dunn's multiple comparisons tests. **(C)** Representative bright-field images of *acvr1b** explants of the indicated genotypes and treatments over time. Black, red, and green arrowheads indicate timely, precocious, and delayed onset of extension, respectively. **(D)** Heparan sulfate (HS) injected embryos display a dorsalized phenotype at the 2-somite stage, indicated by increased animal–vegetal length (red lines). **(E)** Representative bright-field images of *acvr1b** explants of the indicated genotypes and treatments over time, as in (C).

DISCUSSION

Morphogenesis requires precise temporal coordination of cell behaviors to ensure proper tissue shape and function, however, the molecular mechanisms governing its timing remain poorly understood. In this study, we uncover a novel a molecular mechanism controlling the timing of C&E morphogenesis. After establishing that transcription is required at gastrulation onset for C&E, we identified the sulfatase modifying factors *sumf1*/FGE and *sumf2*/pFGE as key temporal regulators. Our data support a model (**Figure 7**) in which, prior to gastrulation, maternally deposited *sumf1*/FGE activates sulfatases enzymes, including the extracellular sulfatase Sulf1. At gastrulation onset, *sumf1* and *sumf2* transcript abundance inverts, and the antagonistic effect of *sumf2*/pFGE on *sumf1*/FGE reduces Sulf1 activity. Consequently, increased levels of sulfated HSPGs promote and/or permit C&E morphogenesis.

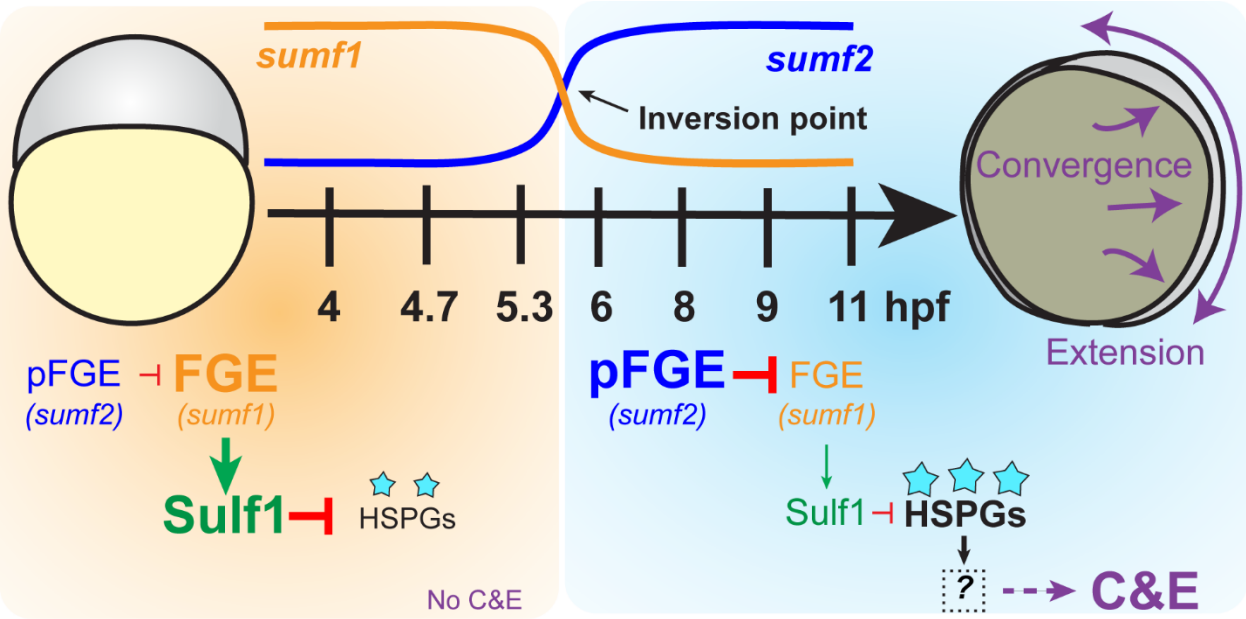

Figure 7. A model for how sulfatase modifying factors regulate the timing of C&E morphogenesis
Before gastrulation (orange), maternally expressed *sumf1*/FGE activates Sulf1, maintaining low levels of 6-*O*-sulfation (cyan stars) on HSPGs. At the onset of gastrulation (light blue), an inversion in *sumf1* and *sumf2* transcript abundance (inversion point) increases the antagonistic action of *sumf2*/pFGE on *sumf1*/FGE, thereby reducing Sulf1 activity. This shift leads to increased 6-*O*-sulfation of HSPGs, generating a permissive environment for C&E morphogenesis via an unknown downstream mechanism.

472 **Developmental roles of sulfatase modifying factors**

473 This is (to our knowledge) the first report of a role for sulfatase modifying factors in vertebrate
474 gastrulation. In humans, mutations in *SUMF1* cause multiple sulfatase deficiency (MSD), a rare and fatal
475 autosomal recessive disorder characterized by lysosomal dysfunction, developmental delay,
476 neurodegeneration, and skeletal defects including scoliosis, facial dysmorphism, and growth retardation (*54,*
477 *93*). Mouse *Sumf1*-/- models exhibit early postnatal lethality (*94*), and MZ*sumf1*-/- zebrafish recapitulate some
478 MSD features such as cranial malformations and early growth retardation (*77*). However, these fish can survive
479 to adulthood, suggesting the presence of alternative mechanisms of sulfatase activation in this species (*77*).
480 Indeed, *Escherichia coli, Caenorhabditis elegans,* and *Sacchromyces cerevisiae* possess sulfatase genes but
481 lack a Sumf1 homolog, suggesting the existence of an alternative formylglycine generating enzyme in these
482 organisms (*55*). Whether a similar alternative enables MZ*sumf1*-/- zebrafish to survive is unknown.

483 The role of *sumf2*, on the other hand, had not been studied in vertebrate development. Like MZ*sumf1*-/-
484 mutants, we found that MZ*sumf2*-/- fish can reach adulthood and some individuals exhibit scoliosis and
485 craniofacial abnormalities (**Figure Suppl. 2E**)**.** This indicates that although *sumf2*/pFGE has a significant role
486 in the timing of C&E morphogenesis, it is not ultimately essential for life in zebrafish. Whether its loss is
487 compatible with life in other vertebrate species remains to be tested. Although Sumf1 orthologs are present in
488 prokaryotes, Sumf2 is restricted to eukaryotes and has been lost in several metazoan clades, including
489 arthropods (*54, 74, 93, 95*). *sumf2*/pFGE and *sumf1*/FGE amino acid sequences are highly similar, but
490 *sumf2*/pFGE lacks formylglycine generating activity due to the absence of an active site (*54, 58, 59*). It was
491 shown in human tissue culture that *sumf2*/pFGE reduces *sumf1*/FGE enzymatic activity (*59, 64*), which is
492 thought to occur through direct physical binding and possible interactions with sulfatase enzymes (*58, 64*). This
493 antagonism is consistent with our findings in zebrafish that MZ*sumf2*-/- phenotypes are exacerbated by *sumf1*
494 OE (**Figure 2**) and co-overexpression of both factors offsets the effects of their individual overexpression
495 (**Figure 3**). Further, MZ*sumf1*-/-; MZ*sumf2*-/- double mutant explants phenocopy the C&E delay observed in
496 MZ*sumf1*-/-, indicating that *sumf1* is epistatic to *sumf2* (**Figure 3**), consistent with a model in which
497 *sumf2*/pFGE governs gastrulation cell movements in its capacity as a *sumf1*/FGE inhibitor.

498

499

500 **Sulf1 and HSPGs regulate gastrulation cell movements**

501 Our data implicate Sulf1 as the primary sulfatase affecting C&E timing downstream of *sumf1*/FGE and
502 *sumf2*/pFGE regulation. Sulf1 catalyzes the removal of 6-*O*-sulfation from the D0S6 and D2S6 disaccharides
503 of HSPGs (*66*), precisely the modifications that were increased between early and late zebrafish gastrulation
504 (**Figure 5**). Notably, this function is also served by the extracellular sulfatases Sulf2a and Sulf2b. However,
505 *sulf2b* OE did not cause pronounced gastrulation phenotypes, and although *sulf2a* OE induced C&E defects in
506 both embryos and explants (**Figure Suppl. 4**), it did not (unlike *sulf1* OE) alter the onset of explant extension.
507 Although these sulfatases target the same substrate, evidence indicates that human Sulf1 and Sulf2 act on
508 distinct polysaccharide substrates (*96*), which may also explain the differential effects of *sulf1* and *sulf2a/b* in
509 zebrafish gastrulae. While the contribution of other sulfatases cannot be excluded, our finding that *sumf1*
510 overexpression no longer affects C&E onset in the absence of *sulf1* strongly implicates Sulf1 as the key
511 regulator of C&E timing. It is less clear that reduced Sulf1 activity is predominantly responsible for the overall
512 increase in HS levels in later gastrulation, as the abundance and sulfation patterns of HS chains are ultimately
513 determined by the activity of multiple enzymes, including glycosyltransferases, heparinases, sulfatases and
514 sulfotransferases (*97*). Interestingly, *xylosyltransferase I* (*xylt1*), whose activity initiates HS biosynthesis *(97),*
515 was also among our candidate 'trigger' genes upregulated at gastrulation onset (**Figure 1**), making it an
516 especially compelling candidate for further investigation.

517 HSPGs and the sulfatases that modify them have long been known to regulate gastrulation
518 morphogenesis. For example, elimination of HS chains or loss of HSPG core proteins glypicans and
519 syndecans caused C&E defects in *Xenopus*, zebrafish, and sea urchin gastrulae (*83, 88, 89, 98-102*). Although
520 our study does not address the HSPG core protein(s) responsible for the observed effects on C&E timing,
521 glypicans and syndecans with established roles in C&E (like *gpc4/kny (100)*) are good candidates. Notably, our
522 manipulations affecting HSPG sulfation tended to produce milder phenotypes that loss of core proteins like
523 Gpc4, consistent with sulfation as a modifier of their activity. Indeed, manipulations that decrease sulfation
524 levels – including overexpression of *sulf1* or *sumf1* mRNA and injection of purified sulfatases into the
525 blastocoel - also disrupted gastrulation in *Xenopus* and sea urchin (*63, 79-81*). Interestingly, loss of sulfatases
526 also caused gastrulation defects (*81, 103, 104*), suggesting that a correct balance of sulfatase activity is
527 required for proper C&E. Our data provide a possible explanation for this: altered timing of C&E movements.

528 We found that sulfatase overexpression both disrupts and delays C&E, but also that both precocious and
529 delayed C&E onset ultimately cause C&E defects (i.e. shorter and wider embryonic axes) in intact embryos.
530 This raises the possibility that previously reported C&E defects upon gain or (especially) loss of sulfatase
531 function could be the result of altered timing (discussed further below).

532 *sulf1* deficiency, however, not only alters the timing of C&E but also prevents full explant extension
533 (**Figure 4, Figure Suppl. 4**). This discrepancy in phenotypic severity between Sulf1 and its key regulator
534 (*sumf1*/FGE) may reflect partially independent regulation of sulfatases during zebrafish gastrulation. As
535 discussed above, C&E defects resulting from altered sulfatase activity are relatively mild in zebrafish, and the
536 mutant embryos examined here can ultimately survive to become fertile adults. This suggests the existence of
537 compensatory mechanisms that support continued primary body axis elongation after gastrulation is complete.
538

539 **Temporal dynamics of gastrulation cell movements**

540 Although it is intuitive that delayed C&E ultimately manifests as a C&E defect *in vivo*, it is less clear how
541 increased and/or precocious convergence movements (as seen in MZ*sulf1*-/-, MZ*sumf1*-/-, and *sumf2* OE
542 embryos) lead to similar phenotypes (**Figure 3 and Figure 4**). We speculate that all gastrulation cell
543 movements – epiboly, internalization, and C&E – must be coordinated in both space and time to properly
544 shape the nascent body axes. If C&E movements are accelerated, they become out of sync with other
545 concurrent cell movements, disrupting axis extension. This would explain why morphological C&E defects are
546 detected in MZ*sumf1*-/- mutants but not explants (**Figure 2 and Figure Suppl. 3**), in which C&E occur in the
547 absence of epiboly and internalization. A similar phenomenon was reported in *Drosophila* gastrulae, in which
548 both slowed and accelerated germ band extension desynchronized morphogenesis between the three germ
549 layers (*105, 106*).

550 It is not yet clear whether sulfatase activity alters the onset of cell movements, their pace, or both. For
551 example, either accelerated cell movements or their precocious onset could manifest as “early onset” of
552 morphological extension within our explants. Indeed, *Drosophila* embryos with loss- and gain-of function
553 mutations in Serotonin signaling components reportedly exhibit slowed and accelerated cell movements driving
554 germ band extension, respectively, without a change in their time of onset (*105-107*). However, our *in vivo* cell-
555 tracking analysis revealed apparent changes in both the onset and speed of convergence movements in

556 zebrafish gastrulae with altered *sumf1, sumf2*, and *sulf1* levels, suggesting that multiple aspects of 'timing' are
557 affected (**Figure 3 and Figure 4**). The precise changes in the timing of cell behaviors upon altered sulfatase
558 activity, and their contributions to our observed phenotypes, are areas of interest for future study.
559

560 **How do HSPGs govern morphogenetic timing?**

561 Cell surface and extracellular HSPGs play key roles in multiple morphogen signaling pathways. For
562 example, HSPGs modulate ligand diffusion and availability (*66, 80, 108, 109*) and receptor binding (*66, 80,*
563 *110*) of morphogens with known roles in C&E, including FGF, Wnt, BMP, and Nodal. HSPGs also regulate PCP
564 signaling through binding of non-canonical Wnt ligands (*98, 100, 110*) and membrane localization of
565 Dishevelled (*80, 98, 99*), which is required for PCP activity (*111, 112*). The structural features of HS chains,
566 particularly their sulfation patterns, are critical for these functions as they determine ligand-binding affinity and
567 selectivity (*97, 113, 114*). Notably, the precise role of HSPG sulfation in signaling is context-dependent and
568 changes throughout development. For example, reduced sulfation inhibits and enhances signaling by Wnt8
569 and Wnt11, respectively (*80, 81, 115, 116*), and HSPGs from different developmental stages have different
570 capacities to bind FGF ligands (*117, 118*). We speculate that sulfatase modifiers may regulate C&E timing by
571 controlling HS sulfation, thereby modulating the degree and dynamics of HSPG-mediated signaling. Indeed,
572 we observed an increase in the levels of sulfated HSPGs (Sulf1 substrates) during late gastrulation (**Figure 5**)
573 and demonstrated that both decreased and increased levels of sulfated HS were sufficient to alter C&E timing
574 (**Figure 6**). Importantly, the phenotypes associated with *sumf1/2* and *sulf1* perturbations reflect disrupted
575 morphogenesis rather than altered cell fate specification, as embryos still give rise to derivatives of all three
576 germ layers, consistent with observations in *Xenopus* and sea urchin gastrulation (*79, 83*). Whether this
577 putative role in morphogen signaling underlies the effect of sulfatase modifiers on C&E timing will be an
578 exciting topic for future studies.
579
580
581

582 **METHODS**

583

584 **Zebrafish**

585 Adult zebrafish were maintained following established protocols (*119*) in compliance with Baylor College of
586 Medicine Institutional Animal Care and Use Committees. Embryos were obtained through natural mating, and
587 staging was based on established morphology (*120*). Fish were chosen from their home tank to be crossed at
588 random, and embryos were randomly chosen from the clutch for injection and inclusion in experiments. WT
589 and mutant embryos were collected in parallel in a period not longer than 10–15 min to minimize
590 developmental differences and raised in egg water at 28.5°C under identical conditions. Experiments on WT
591 embryos were conducted in either the AB or TU background, depending on the background of the mutant line
592 used. The mutant lines used were *sum*^{la015919Tg} (ZFIN ID: ZDBALT-120806-11568), *sulf1*^{sjr9} (Kaur et al,
593 submitted), *sumf2*^{bcm126} (this study, described further below) and *oep*^{tz257} *(100). oep-/-* embryos were rescued to
594 viability by injection of 50 pg of *oep* mRNA *(121)* and raised to adulthood, then intercrossed to generate
595 MZ*oep-/-* embryos for explantation. MZ*sumf1*-/-; MZ*sumf2*-/- double mutants were generated by crossing
596 single homozygous mutants to obtain double heterozygotes, which were subsequently incrossed.

597

598 ***sumf2*^{bcm126} generation and genotyping**

599 *Sumf2* zebrafish mutants were generated using the CRISPR–Cas9 system. A single guide (sg)RNA targeting
600 exon 2 of *sumf2* (5′-GGATGGAGAATCGCCAACAC-3′) was designed using CRISPRscan (*122*). sgRNAs were
601 transcribed using T7 RNA polymerase (NEB, M0251S) from DNA templates generated with the forward primer
602 (Fw): gaaattaatacgactcactataGGATGGAGAATCGCCAACACgttttagagctagaaatagc, and the reverse primer
603 (Rv): aaaagcaccgactcggtgccactttttcaagttgataacggactagccttattttaacttgctatttctagctctaaaac. 1 µl of gRNA was pre-
604 incubated for 10 min at 37 °C with Cas9 protein (NEB, M0646M) and 300mM KCl (as described by (*123*)). AB
605 WT Embryos were injected at the single-cell stage with 1 nl of the gRNA–Cas9 complex and cutting efficiency
606 was assessed using a T7 endonuclease I assay (*124*). A PCR fragment encompassing the target sequence
607 was amplified from genomic DNA (gDNA) of individual embryos (using the following primers: Fw:
608 AGATGGTGTTTATTCCTGGTGG, Rv: TCCTCTGATACAAAATCCTGGAA) and incubated with T7
609 endonuclease I (NEB #M0302L) in NEB 10× Buffer 2. Injected F0 embryos were raised and outcrossed to AB

610 WT fish. F1 progeny were genotyped by Sanger sequencing to identify transmitted mutations. A 7 bp insertion
611 in exon 2 of *sumf2* (sumf2^{bcm126}), resulting in an early stop codon, was selected (**Figure Suppl 2A**). This
612 mutation introduced an XcmI restriction site, enabling genotyping by digestion of the PCR-amplified target
613 fragment with XcmI (NEB, R0533S) (**Figure Suppl 2B**). Heterozygous F1 carriers were incrossed to produce
614 the F2 generation. Homozygotes F2 fish were raised and incrossed to obtained MZ*sumf2*-/- embryos for
615 experiments.
616

617 **RT-qPCR**

618 RNA was isolated from 50 pooled WT and MZ*sumf2*-/- 24 hpf larvae from three independent clutches. Total
619 RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, 15596018), purified by sodium acetate
620 precipitation, eluted in 10 mM Tris pH7.5 and treated with Turbo DNAase I (Invitrogen, AM2238) at 37°C for 30
621 min. For each sample, 1μg of the RNA was reverse-transcribed using iScript Reverse Transcription Supermix
622 for RT-qPCR (Biorad, #1708840). *sumf2* specific primers were designed to span exon-exon junctions [Exon 1-
623 2 (pair 1) FW: CACAGTGTCTTGTGCAGCAG, RV: AGTTGGAGTTGGTGACAGGA; Exon 3-5 (pair 2) FW:
624 GGCTGAAACATTTGGCTGGA, RV: GGCATCATTCCAGCTGACCT]. *rpl13a-1* was used as a housekeeping
625 gene [FW: TCTGGAGGACTGTAAGAGGTATGC, RV: AGACGCACAATCTTGAGAGCAG]. qPCR was
626 performed in technical triplicates using SsoAdvanced Universal SYBR Green Supermix (Biorad, #1725270).
627 Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.
628

629 **Preparation and microinjection of mRNA**

630 All mRNAs were transcribed using the SP6 mMessage mMachine Kit (Fisher Scientific, AM1340) and purified
631 using Bio -Rad Microbiospin columns (Bio-Rad, 7326250). Single-celled embryos were placed in agarose
632 molds (Adaptive Science Tools, I-34) and injected with 0.5-1 nl volumes using pulled glass needles (Fisher
633 Scientific, 50-821-984). Doses of mRNA per embryo were as follows: 0.5 pg *acvr1b** (*68*), 10 pg *ndr2* (*125*),
634 50-100 pg s*umf1*, 100 pg *sumf2*, 500 pg *sfGFP,* 500 pg *sulf2b,* 500 pg *sulf2a,* 500pg *gnsb,* 50-500 pg *sulf1,*
635 500 pg *sgsh,* 500 pg *arsb,* 500 pg *gnsa,* 500 pg *ids,* 500 pg *galns,* 500 pg *arsa,* 500 pg *sts,* 100 pg *mem-GFP*
636 and 100 pg *H2B-scarlet.* Templates for *sumf1*, *sumf2*, and all sulfatases were generated by Gibson cloning

637 (*126*) each of their open reading frames (synthesized by Twist Biosciences) into a PJS2 vector linearized with
638 EcoRI.
639
640 **Whole mount *in situ* hybridization (WISH)**
641 *tbxta (brachyury, t)*, *dlx3b* and *egr2b (krox20)* antisense riboprobes were transcribed using NEB T7 RNA
642 polymerase (NEB, M0251s) and labeled with digoxigenin NTPs (Sigma/Millipore, 11277073910) NTPs
643 (Sigma/Millipore, 11685619910). WISH was performed according to (*127*). Embryos were fixed overnight in at
644 4°C 4% PFA in PBS, rinsed in PBS + 10% tween-20 (PBT), and dehydrated into methanol. Following
645 rehydration into PBT, embryos were pre-incubated for at least two hours in hybridization buffer with 50%
646 formamide (in 0.75 M sodium chloride, 75 mM sodium citrate, 0.1% tween 20, 50 mg/mL heparin (Sigma), and
647 200 mg/mL tRNA) at 70°C, and hybridized overnight at 70°C with antisense probes (1-5 ng/mL) in hybridization
648 buffer. Samples were washed gradually into 2X SSC buffer (0.3 M sodium chloride, 30 mM sodium citrate),
649 and then gradually from SSC to PBT. Samples were blocked at room temperature for several hours in PBT
650 with 2% goat serum and 2 mg/mL bovine serum albumin (BSA), then incubated overnight at 4°C with anti-DIG
651 antibody (Roche #11093274910) at 1:5000 in block. After extensive washes in PBT, embryos were rinsed in
652 staining buffer (PBT +100 mM Tris pH 9.5, 50 mM $MgCl_2$, and 100 mM NaCl) and developed in BM Purple AP
653 substrate (Roche) until the desired staining intensity was achieved. Staining was stopped with 10 mM EDTA in
654 PBT before imaging.
655
656 **Blastoderm explants**
657 Blastoderm explants were prepared as described by (*128*). Briefly, uninjected, *acvr1b*-,* or *ndr2*-injected
658 embryos were dechorionated at the 256-cell stage using pronase (Roche; 1 ml of 20 mg/ml stock in 15 ml 3×
659 Danieau's solution). At the 512-cell stage, approximately 1/3 of the most animal blastoderm cells were excised
660 using Dumont #55 forceps (Fisher Scientific, NC9791564) on an agarose-coated plate containing 3× Danieau's
661 solution. Explants were allowed to heal briefly before being transferred into agarose-coated 6-well plates
662 containing explant medium [Dulbecco's modified eagle medium with nutrient mixture F-12 (Gibco 11330032)
663 containing 2.5 mM L-glutamine, 15 mM HEPES, 3% newborn calf serum (Invitrogen 26010–066), 50 units/mL
664 penicillin, and 50 mg/mL streptomycin (10,000 U/mL pen-strep at 1:200, Gibco 15140163)]. Explants were

665 incubated at 28.5 °C until sibling embryos from the same genetic background reached the desired
666 developmental stage.
667
668 **Alcian Blue staining**
669 Embryos were fixed in PFA 4% overnight, extensively washed in PBS, and incubated in Alcian Blue staining
670 solution pH 1 (0.2% Alcian Blue 8 GX (Sigma # A5268), 50% ethanol, ~0.1N HCl) for 48 h at room
671 temperature, protected from light. Samples were then gradually rehydrated into PBS, post-fixed in 4% PFA,
672 washed with PBS, and rinsed in 2% KOH. Embryos were cleared through a graded series of glycerol in 2%
673 KOH (20%, 40%, 60%) and stored in 80% glycerol in 2% KOH for imaging and long-term storage.
674
675 **Transcription inhibitor treatment**
676 1 μM Triptolide (Sigma # T3652) or an equivalent volume of DMSO was added to the media of blastoderm
677 explants in agarose-coated 6-well plates at 50% (4.7 hpf), shield (6 hpf), 70% (7.5 hpf) or 80% (8.5 hpf). 0.5
678 μM Flavopiridol (Selleck, S1230) or an equivalent volume of DMSO was added to the media of blastoderm
679 explants at 50% (4.7 hpf) and washed-out twice with 0.3x Danieau solution, before incubation with fresh
680 explant medium.
681
682 **Sodium Chlorate treatment**
683 Dome stage (4.33 hpf) embryos or blastoderm explants were treated with 200 or 50 mM of Sodium Chlorate
684 (VWR # 7775-09-9) in egg water or explant medium.
685
686 **Heparan Sulfate injections**
687 Uninjected or *acvr1b**-injected embryos were dechorionated at the 64-cell stage using pronase (Roche; 1 mL
688 of 20 mg/mL stock in 15 mL 3× Danieau's solution). Embryos were transferred to agarose-coated plates
689 containing cubical depressions (Adaptive Science Tools, PT-1) filled with 0.3× Danieau's solution. At the 256-
690 cell stage, embryos were injected with 2 nL of 5 ng/mL heparan sulfate (Sigma-Aldrich, H7640) or nuclease-
691 free water into the extracellular space.
692

693 **Microscopy**

694 Live embryos injected with *H2B-mScarlet* and *mem-GFP* mRNA were manually dechorionated and mounted in
695 0.3-0.35% low-melt agarose (Thermo Fisher Scientific, 16520100) in glass-bottomed 35 mm Petri dishes
696 (Fisher Scientific, FB0875711YZ) for imaging using a Nikon ECLIPSE Ti2 inverted confocal microscope
697 equipped with a Yokogawa W1 spinning disk unit, PFS4 camera, and 405/488/561 nm lasers (emission filters:
698 455/50, 525/36, 605/52). Temperature was maintained at 28.5°C during imaging using a Tokai Hit STX stage
699 top Incubator. For live time-lapse series, 100 µm z-stacks with a 2 µm step were collected every 5 minutes for
700 5 hours using a Plan Apo Lambda 20x dry objective lens. Live blastoderm explants were mounted in rounded
701 chambers made in 1% low-melt agarose in glass-bottomed dishes containing explants medium and imaged in
702 the same scope. For live time-lapses, 14 µm z-stacks were obtained with a 2 µm step every 10 minutes for 6
703 hours using a Plan Apo Lambda 10x dry objective lens. Images of WISH and alcian blue-stained embryos and
704 live embryos, larvae and explants were taken with a Nikon Fi3 color camera on a Nikon SMZ745T
705 stereoscope.

706

707 **Image analysis**

708 ImageJ/Fiji was used to visualize and measure all microscopy data sets.

709

710 *Morphometric analyses*

711 During analysis, researchers were kept unaware of the conditions of all image data using the blind_renamer
712 Perl script (https://github.com/jimsalterjrs/blindanalysis) (blindanalysis: v.1.0.) prior to analysis. To measure the
713 length/width ratios of explants, the length of a segmented line drawn along the midline of each explant
714 (accounting for curvature) was divided by the length of a perpendicular line spanning the maximal width of the
715 explant. Tailbud morphometries were performed in whole-mount embryos staged using *egr2b* expression at the
716 future mid-hindbrain boundary. Notochord width was quantified in dorsal-view images as the mediolateral
717 extent of the *tbxta* expression domain at the midline. Anteroposterior (AP) axis length was measured in lateral-
718 view images from the anterior to posterior boundaries of the *dlx3b* expression domain.

719

720

721 *Explant onset of extension*

722 The onset of explant extension was assessed in a blinded manner as the time point when a visible tip first
723 emerged from an initially rounded explant. Explant were staged according to sibling intact embryos from the
724 same genetic background cultured in the same plate.

725

726 *Cell tracking analysis*

727 Automated nuclear tracking was performed using the ImageJ TrackMate7 plugin (*129*) in the dorsal
728 hemisphere (encompassing dorsal and lateral cells) of zebrafish gastrulae injected with *H2B-scarlet* mRNA.
729 TrackMate generated color-coded trajectories and measurements of mediolateral (ML) displacement. Embryos
730 were staged relative to formation of the second somite. To minimize noise from reduced convergence
731 movements near the dorsal midline, cells within 100 µm of the midline were excluded from the analysis.
732 Average displacement in the X (ML) dimension was calculated per time frame, smoothed using a sliding
733 window of four time points, and plotted over time using GraphPad Prism 10.

734

735 **GAG isolation and disaccharide analysis.**

736 Whole zebrafish gastrulae (5.3 or 8.5 hpf, 100 embryos/sample) were homogenized and lysed in 0.5% CHAPS
737 lysis buffer (50 mM HEPES, 120 mM NaCl, 2 mM EDTA, pH 7.4) containing a protease inhibitor cocktail (Roche).
738 50 µL of cell lysate was set aside for protein quantification via BCA assay. Homogenates were diluted 1:10 in a
739 wash buffer (50 mM sodium acetate, 200 mM NaCl, 0.1% Triton X-100, pH 6.0) and incubated with Pronase (0.4
740 mg/ml, Sigma) overnight at 37 °C with mild agitation. The product was centrifuged (4,000 xg, 20 minutes) then
741 passed through a DEAE-Sephacel (Cytiva) column equilibrated in 50 mM sodium acetate buffer, pH 6.0,
742 containing 200 mM NaCl, and desalted using a PD-10 desalting column (Cytiva). The purified GAGs were
743 subsequently enzymatically depolymerized with 2 mU each of heparin lyases I-III (IBEX) and differentially mass
744 labeled by reductive amination with aniline, as described (*86*). Tagged HS disaccharides were analyzed by HILIC-
745 Q-TOF-MS on a Waters Synapt XS Q-TOF mass spectrometer, as previously described (*86*). Samples were
746 quantified using isotopically labeled internal disaccharide standards and normalized to total protein, as measured
747 by BCA.

748

749 **Statistical analysis**

750 Number of embryos (n) and independent experimental replicates (N) for animal studies are stated in graphs. All
751 experiments were performed at least in triplicates. GraphPad Prism 10 software was used to perform statistical
752 analyses and generate graphs for all data analyzed. Datasets were tested for normality prior to analysis and
753 statistical tests were chosen accordingly. The statistical tests used for each data set are noted in figure
754 legends.

755

756 **ACKNOWLEDGEMENTS**

757 We thank Dr. Lila Solnica-Krezel for sharing plasmids and WISH probes, the BCM Center for Comparative
758 Medicine for taking excellent care of our fish, and the Zebrafish International Resource Center for preserving
759 and distributing fish lines used here and by countless members of the community. Thanks also to all members
760 of the Williams lab for their help and feedback on this project.

761

762 **COMPETING INTERESTS**

763 The authors declare no competing interests.

764

765 **AUTHOR CONTRIBUTIONS**

766 A.S.C. and M.K.W. conceived of the project. A.S.C. and M.K.W. performed zebrafish experiments, R.J.W. and
767 A.B. performed HS disaccharide profiling, and R.M.J. and G.K.B. generated the *sulf1* deletion line. S.G. and
768 C.C. performed bioinformatic analysis. A.S.C and M.K.W. wrote the original manuscript. All authors reviewed
769 the manuscript.

770

771 **FUNDING**

772 This work was supported by NIH/NICHD grants R00HD091386 and R01HD104784 to M.K.W. R.J.W. is
773 supported by NIH grant R35GM150736. The glycosaminoglycan disaccharide analyses performed at the
774 CCRC were partially supported by NIH grant R24GM137782 to Parastoo Azadi. G.K.B. was supported by a
775 Bourses d'excellence (Université de Montréal) and a FRQ Doctoral Scholarship. R.M.J. is supported by CIHR
776 grants (PJT-178037, PJT-204048) and FRQS J1 and J2 awards. S.G. and C.C. were partially supported by

CPRIT RP210227 and RP200504, NIH/NCI P30 shared resource grant CA125123, NIH/NIEHS P42 ES027725 and P30 ES030285. Data analysis was performed on the HPC cluster that is managed by the Biostatistics and Informatics Shared Resource (BISR) and supported by an NIH S10 Shared Instrument Grant S10-OD032185, NCI P30-CA125123 and Institutional funds from the Dan L Duncan Comprehensive Cancer Center and Baylor College of Medicine.

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