Supporting Information

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SI Text

Zebrafish Strains. All WT fish pairs used to generate embryos for this study were determined to have low backgrounds of L/R patterning defects. The WT strains used in these experiments include Tu, Alb/+, WIK, and AB. The following additional strains were used in this report: *Tg*(*cmlc2:egfp*) (1) and *Tg*(*cmlc2:dsredt4)* kindly provided by Heather Riley and Deborah Yelon.

Time-Lapse Analysis. Embryos were screened for GFP expression and mounted as follows: The embryo was placed on the coverslip bottom of a round dish and mounted in a large drop of 1% agarose. The embryos were oriented tail down, such that the heart was lying flat and located directly on the top of the embryo. The tip of the notochord was used to aid in orientation. A drop of embryo medium with MESAB was placed on top of the agar-mounted embryo.

The slide was placed on a heated stage set at 29° on an inverted Leica SP5 spectral confocal microscope. The embryo was imaged using a X20 dry objective. To be able to generate 3D reconstructions of the embryos, the confocal was set to collect images from just above the first visible GFP-expressing cells to just below the last visible GFP-expressing cells. In general, 30–40 focal planes were collected at an interval of 1.5–2.5 μm. Images were collected at 2- to 3-min intervals. In general, embryos were imaged from between 18–22 somites until the direction of cardiac jog became apparent. The age at the start of the time lapse depended most on the intensity of the GFP, which can be variable during these early stages. Imaging was initiated once the intensity of the GFP was sufficient.

For 4D analysis, the data were imported into a Volocity (Improvision) library. An image sequence was made for each data set. The image appearance (brightness, contrast, opacity) was modified to optimize the visualization of the region of interest. In some cases, the intensity of the cells was variable such that the cell in one region were very bright while the cells in another region were very dim; if the image was optimized for the dim cell, then the cellular resolution for the bright region was lost. Thus, different images were taken for each type of analysis, and in some cases several image settings were needed to complete the analysis.

Examination of the cross-section of high-resolution confocal images indicates that myocardium can be conceived as a 2D tissue because the tissue is composed of a relatively flat, single cell layer throughout the stages of the time lapse. Thus, as we track the movement of the cells, we are only concerned about the X and Y location of the center of the cells. We determined the visual center of each cell at each time point, modifying the image intensity as needed, and measured the location of that center point by using Volocity. At the first time point, the pixel at the center of the cell was selected by using the magic wand tool set to a tolerance of 1, which ensured that only a single pixel was selected. The center of the same cell was marked in the next time point until the end of the data set. Each cell center was measured as an X and Y location. The set of cell locations was then turned into a track by selecting all of the measurements for that cell and having Volocity connect these center points (termed Manual Track). The XY locations of the cells and the cell tracks can be imaged and overlaid on the high-resolution confocal images. In this way, we can visualize the movements of each cell in the myocardium.

Fate Mapping Analysis. *Tg*(*cmlc2:dsredt4)* embryos were injected with the Tg(cmlc2:Dendra) plasmid and transposase RNA alone or in conjunction with the swt morpholino or spaw morpholino directly into the cell at the one- or two-cell stage. Embryos were screened at 18 hpf, and those exhibiting left- or right-restricted Dendra expression were separated for further analysis. These embryos were then imaged on the SP5 spectral confocal microscope at 18–20 hpf, mounted as described for time-lapse analysis, removed from agarose, and maintained individually. These embryos were subsequently imaged again at 24–28 hpf and 48 hpf and were mounted as previously described but in a ventral orientation. In these experiments, both embryos heterozygous and homozygous for the dsRED insertion were used. Red fluorescence in homozygotes is significantly brighter than in heterozygotes. Thus in heterozygotes, the intensity from our *cmlc2:Dendra* plasmid often overwhlemed the dsRED signal, and merged cells appeared green instead of yellow as they did in homozygotes.

Morpholino and cmlc2:Dendra Injections. RNA and plasmid injections were performed as previously described (2). For creation of the *cmlc2*:Dendra plasmid, the *cmlc2* promoter was removed from the pDestTol2CG2 plasmid (3) by restriction digest with *Hin*dIII and *Nco*I enzymes. The promoter was then inserted into the *Eco*RV site of the multiple cloning region in the pTol2005b plasmid (5) to create BL#414. The Dendra sequence was PCR amplified from the pDendra 2-C plasmid (Evrogen) by using primers F-5′-AGGTGCCTCGAGATGAACACCCCGGG-3′ with the addition of an NheI restriction site, and R-5′-GATACGCATATGACACCTGGCTGGGCAGGG-3′ with the addition of an *Apa*I restriction site. The Dendra sequence was subsequently inserted between the *Nhe*I and *Apa*I sites within the pTol2005b-*cmlc2* plasmid to create BL#415. BL#415 was injected directly into the cell at the 1- to 2-cell stage with transposase RNA alone or in combination with either 9 ng of the *swt* morpholino (5′-ATGCACTGTAATTTACCAAGTCAGG-3′) or 1 ng of the *spaw* morpholino (5′-GCACGCTATGACCG-GCTGCATTGCG-3′ (4). The *swt* morpholino (J. Sullivan-Brown and R.D.B., unpublished work) (GeneTools, LLC) covers the exon 5 intron 5 splice junction, whereas the *spaw* morpholino recognizes the start site for protein translation.

1. Huang CJ, Tu CT, Hsiao CD, Hsieh FJ, Tsai HJ (2003) Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev Dyn* 228:30–40.
2. Gritsman K, *et al.* (1999) The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* 97:121–132.
3. Kwan KM, *et al.* (2007) The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* 236:3088–3099.
4. Long S, Ahmad N, Rebagliati M (2003) The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development* 130:2303–2316.
5. Villefranc JA, Amigo J, Lawson ND (2007) Gateway compatible vectors for analysis of gene function in zebrafish. *Dev Dyn* 236:3077–3087.

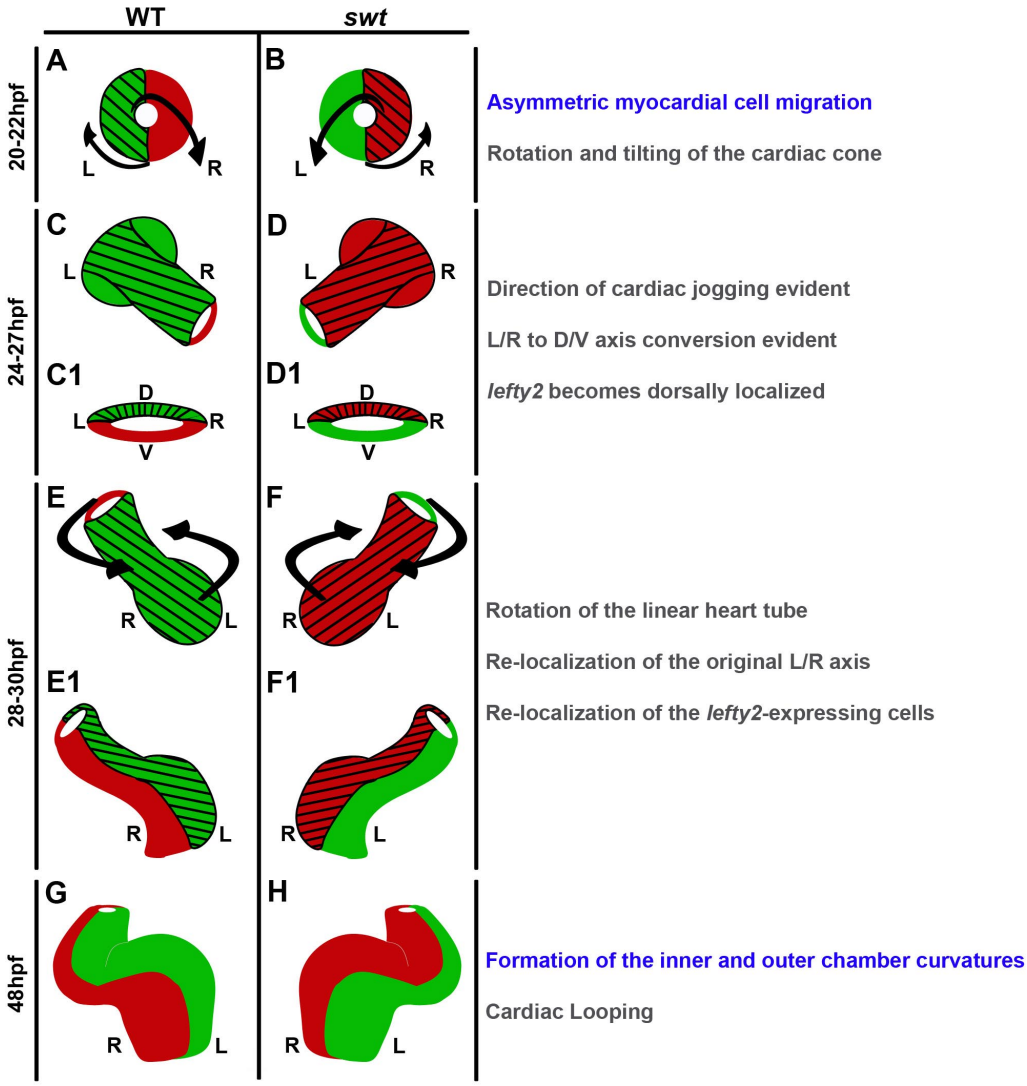
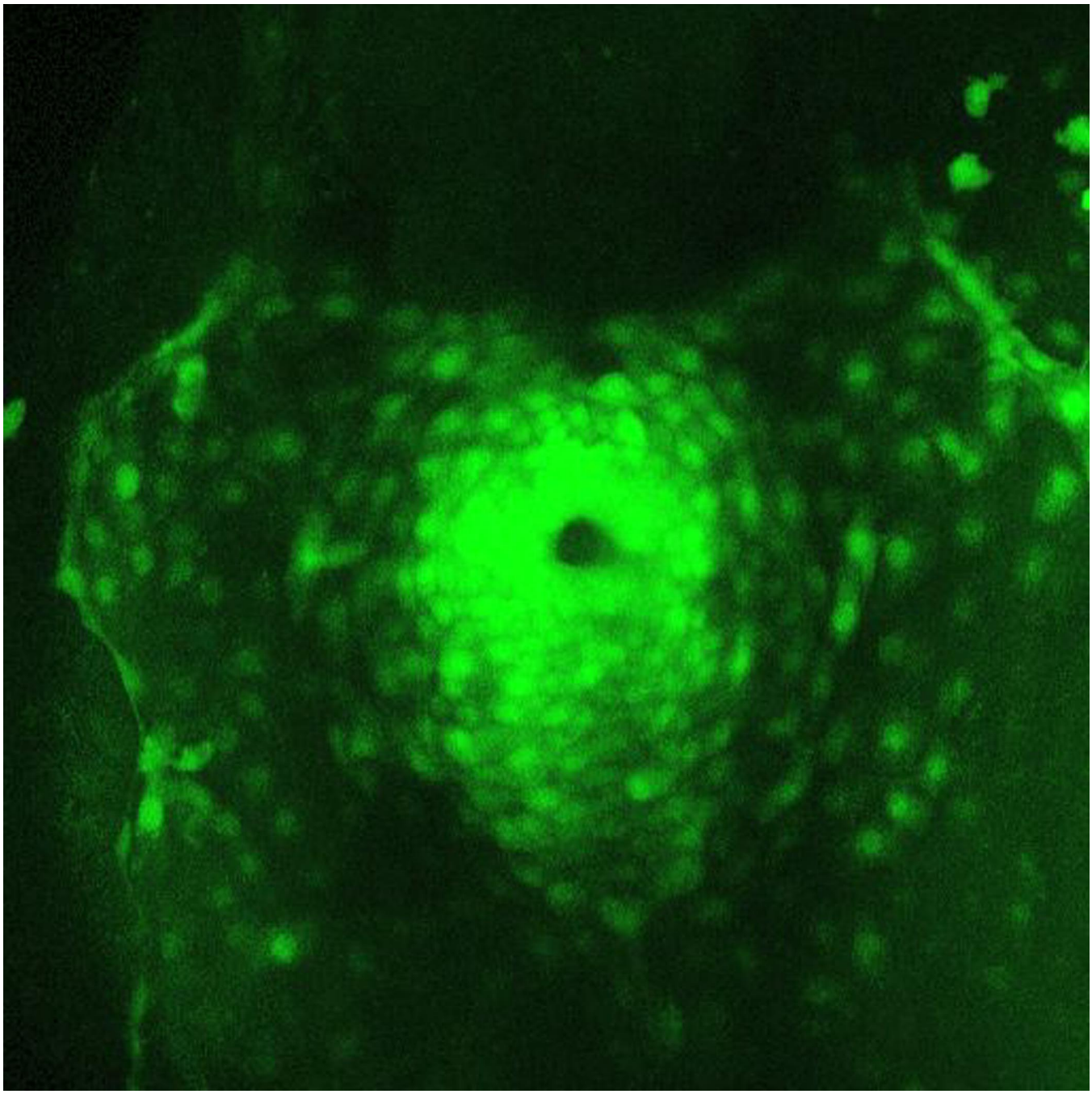
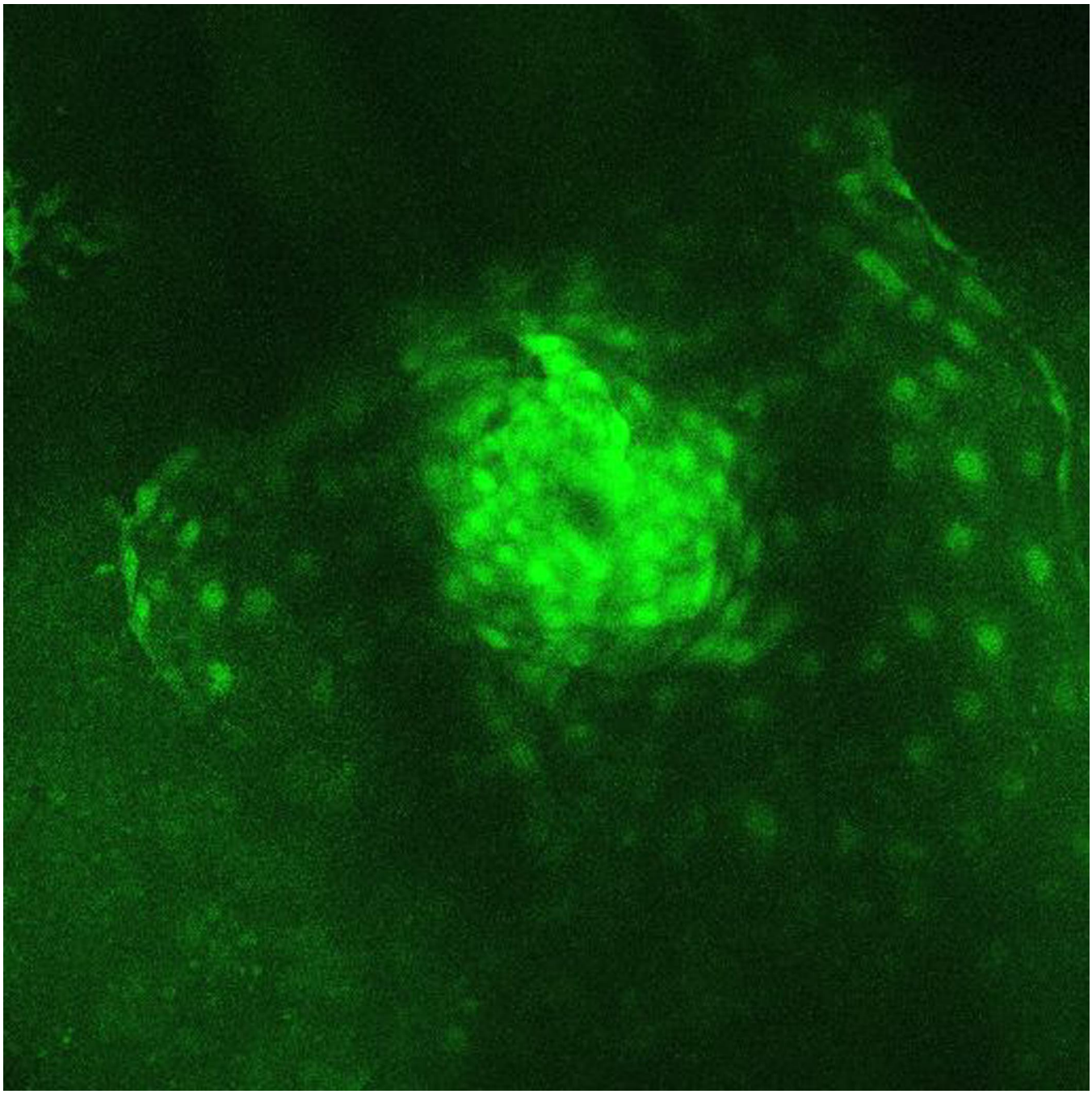


Fig. S1. Direct and indirect roles for Nodal signaling during establishment of cardiac L/R asymmetries. Diagrams of the heart from 20–48 hpf, with the left cardiac field in green and the right cardiac field in red and black hatchmarks to indicate the domains of *lefty2* expression. (*A*–*D*) The heart is viewed dorsally. (*C1* and *D1*) The heart is viewed in cross-section. (*E*–*H*) The heart is viewed ventrally. Blue type indicates morphogenetic events that we believe to be directly influenced by Nodal signaling, whereas gray type indicates events that are indirectly affected by asymmetric gene expression (*A*). In WT embryos, asymmetric myocardial cell migration toward the anterior and left promotes a clockwise rotation of the cardiac cone and the subsequent repositioning of the cone from extension along the D/V axis to the A/P axis. The L/R directionality of asymmetric cell migration, cardiac-cone rotation, and cone tilt are all reversed in a subset of *swt* morphants (*B*). The laterality of Nodal signaling is likely to directly influence the direction of cell migration along the L/R axis, thereby indirectly determining the directions of cardiac cone rotation and tilt. In WT embryos, the indirect consequence of these asymmetric cell migrations within the cardiac cone is a left-directed jog evident by 24 hpf (*C*). The combination of cone rotation and tilt results in the original left cells becoming relocalized to the dorsal side of the heart tube during cardiac jogging (*C1*). In ≈30% of *swt* morphants, reversed L/R directionality of cell migration, cone rotation, and cone tilt likely results in a reversed, right-directed cardiac jog evident at 24 hpf (*D*). In these embryos, right-derived cells are now localized to the dorsal side of the heart tube, with these right cells expressing *lefty2* (*D1*). Importantly, in both WT embryos with left jog and *swt* morphants with right jog, the *lefty2*-expressing cells become dorsally localized, regardless of where they originated along the L/R axis within the LPM (*C1* and *D1*). In both WT and *swt* morphants, these *lefty2*-expressing cells remain localized to the dorsal side of the heart tube through 28 hpf (*E* and *F*). Between 28–30 hpf, the heart tube in WT embryos undergoes a left-directed rotation which repositions the original left cells to the left side of the heart tube at the onset of cardiac looping (*E1*). As the original left cells in these embryos were exposed to Nodal signaling, *lefty2* expression is also restricted to the left side of the heart tube (*E1*). In both *swt* morphants and *spaw* morphants with right jog, the rotation occurring at 28–30 hpf is reversed around the L/R axis and is directed toward the right (*F*). Although this still results in the original left cells becoming repositioned to the left side of the heart, in *swt* morphants the right, rather than left, myocardium had likely been exposed to Nodal signaling. This results in the *lefty2*-expressing cells being positioned to the right at the onset of cardiac looping in these morphants (*F1*). As heart-tube rotation occurs with consistent directionality in all embryos with left jog and with reversed directionality in all embryos with right jog, regardless of genotype, the direction of this rotation appears to be directly determined by the laterality of cardiac jog. In WT embryos, dextral cardiac looping is evident by 48 hpf with the original left cells consistently contributing to the inner curvature of the ventricle and the outer curvature of the atrium (*G*). In *swt* morphants with right-directed jog, the direction of cardiac looping is reversed in nearly 100% of embryos, with the ventricle positioned to the left of the atrium (*H*). In these morphants, the original left cells now contribute to characteristic ''right'' structures, giving rise to the outer curvature of the ventricle and the inner curvature of the atrium (*H*). Given this, along with the randomization in the L/R position of chamber curvature formation observed in the right-jogged hearts of *spaw* morphants, we believe Nodal signaling to directly bias the location of inner ventricular and outer atrial chamber curvature formation, thereby indirectly determining the direction of cardiac looping. L, left; R, right; D, dorsal; V, ventral.



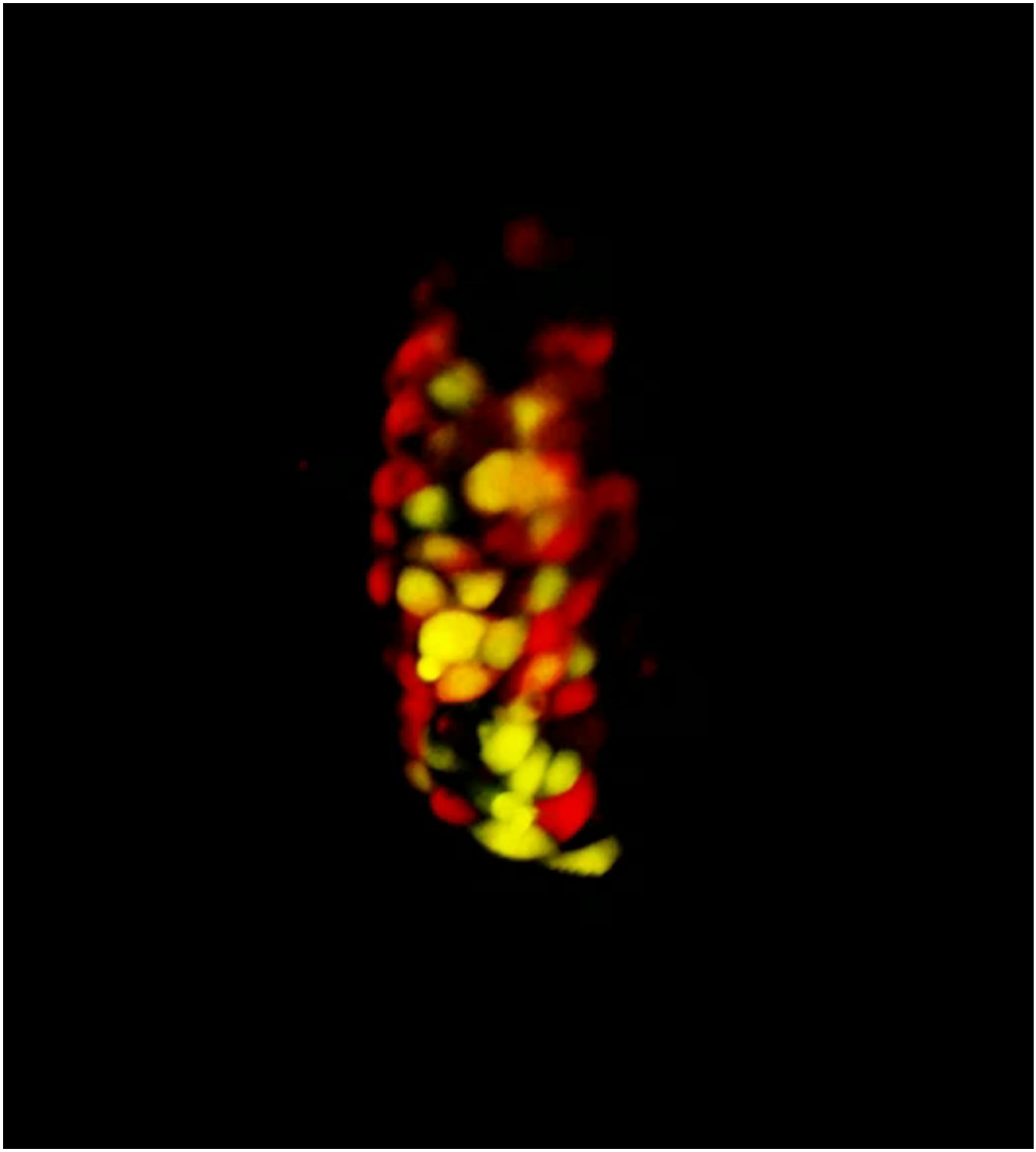
Movie S1. Time lapse of left-directed asymmetric myocardial cell migration in a WT embryo. Dorsal view of the myocardium in a *Tg*(*cmlc2:egfp*) embryo. Anterior toward the top. Movie extends over 70 min of development between 18–21 hpf. The left atrial myocardium migrates along the lateral edge of the cardiac cone toward the anterior and left of the embryo. Those atrial cells originally in the posterior of the cone exhibit the greatest displacement over time. Right atrial myocardium also migrates asymmetrically to the anterior and left but with trajectories directed toward the center of the cone rather than along the lateral edge. This coordinated, asymmetric migration results in a leftward displacement and clockwise rotation of the entire cardiac cone.

Movie S1 (MOV)



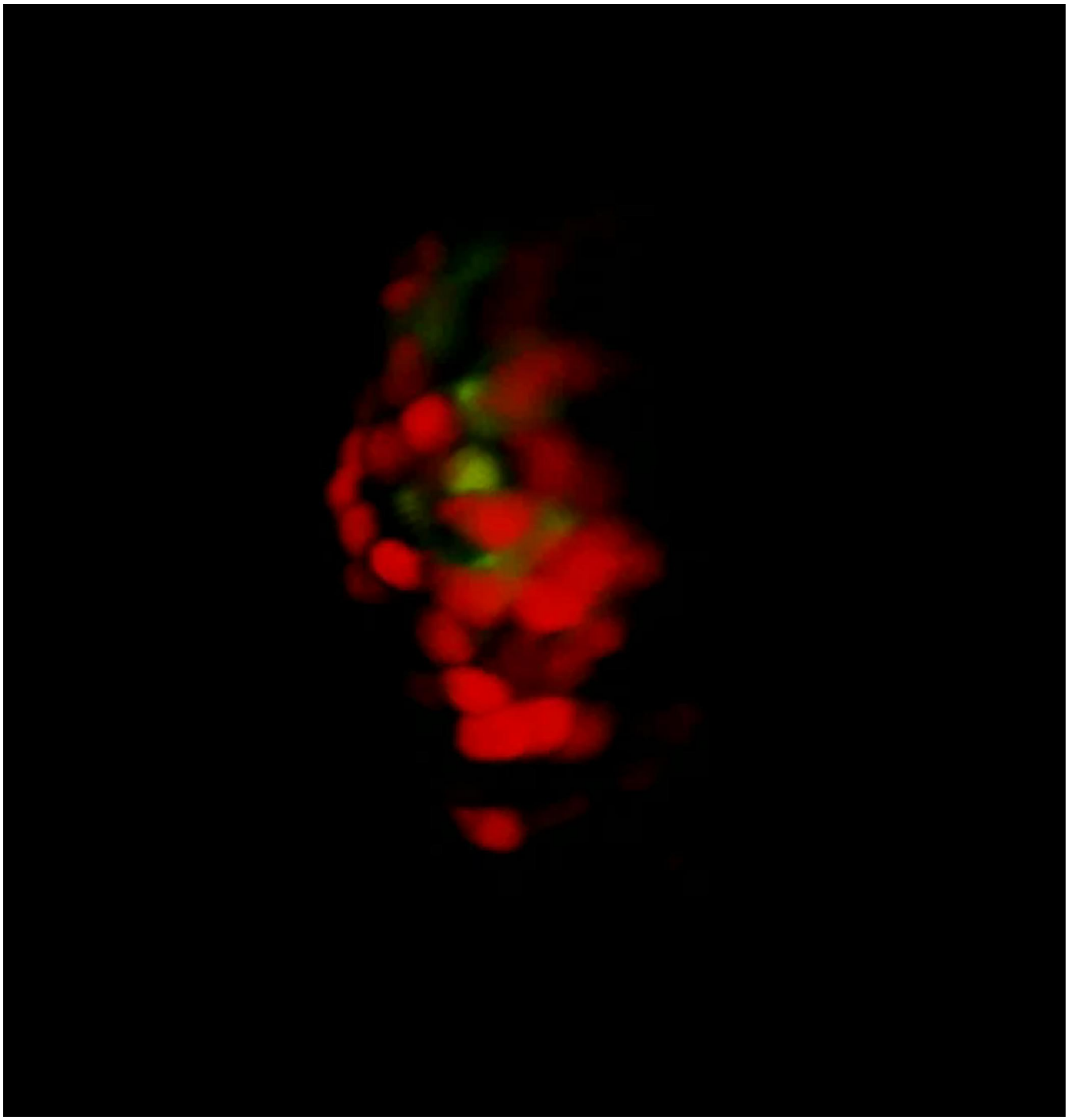
Movie S2. Time lapse of right-directed asymmetric myocardial cell migration in a *swt* morphant. Dorsal view of the myocardium in a *Tg*(*cmlc2:egfp*) embryo injected with *swt* morpholino. Anterior toward the top. Movie extends over 70 min of development between 18–21 hpf. In contrast to WT, this *swt* morphant displays migration of the right atrial myocardium along the lateral edge of the cardiac cone toward the anterior and right. Left myocardial cells also display a right-directed migration toward the center of the cardiac cone. These movements result in a rightward displacement and counterclockwise rotation of the cone structure. We note that the specific movement of cells appears slightly different in *swt* morphant, likely as a consequence of the mutant phenotypes.

Movie S2 (MOV)



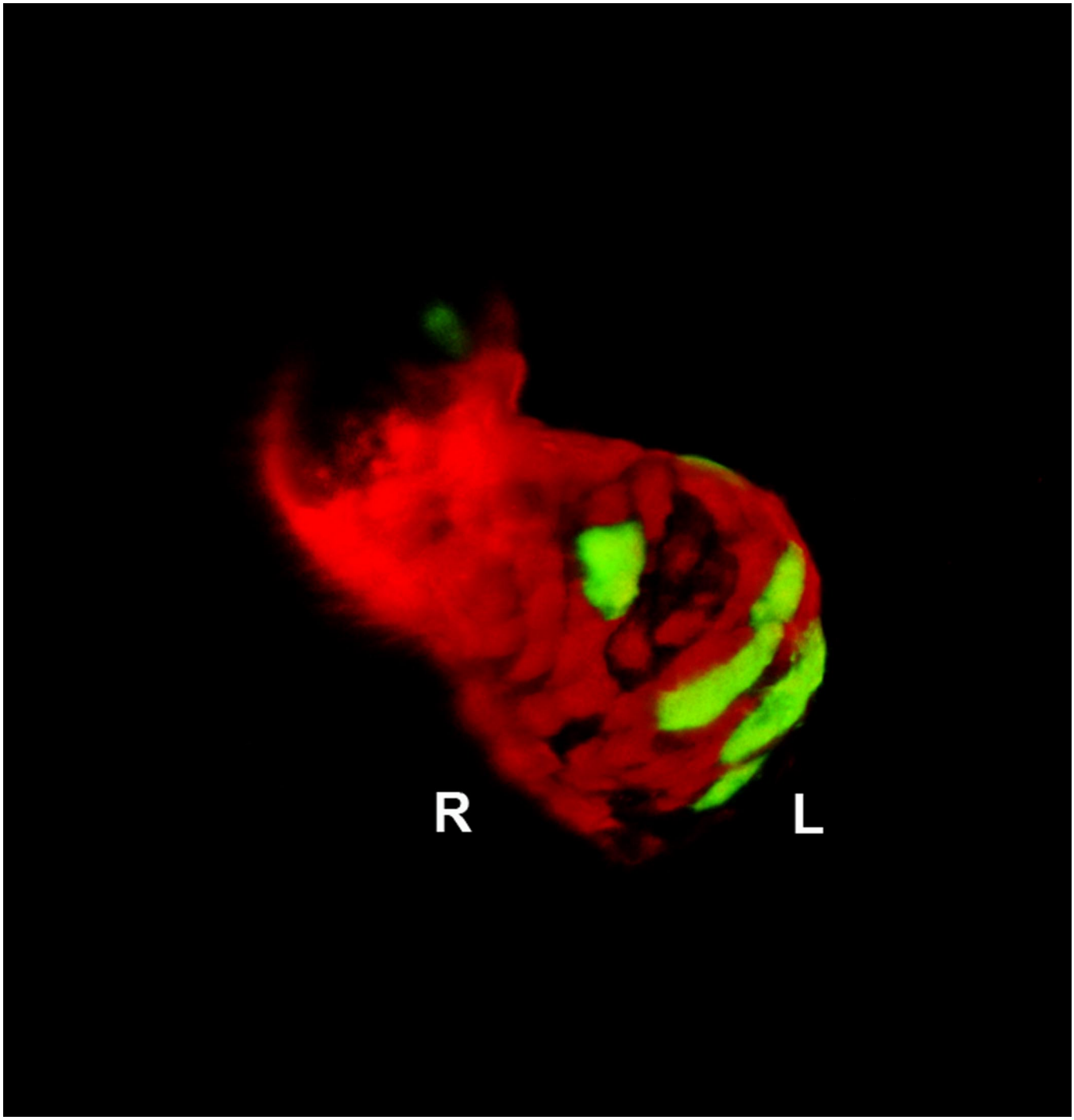
Movie S3. Original left myocardial cells of WT embryos become localized to the dorsal side of the linear heart tube by 24 hpf. Dorsal view of a WT, left-jogged heart tube at 24–28 hpf, with ventricle positioned to the top and atrium to the bottom, of a *Tg*(*cmlc2:dsredt4*) embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the original left cardiac cells. A 360° rotation of the heart tube about the L/R axis reveals the restriction of these GFP-expressing cells to the dorsal region of the linear heart.

Movie S3 (MOV)



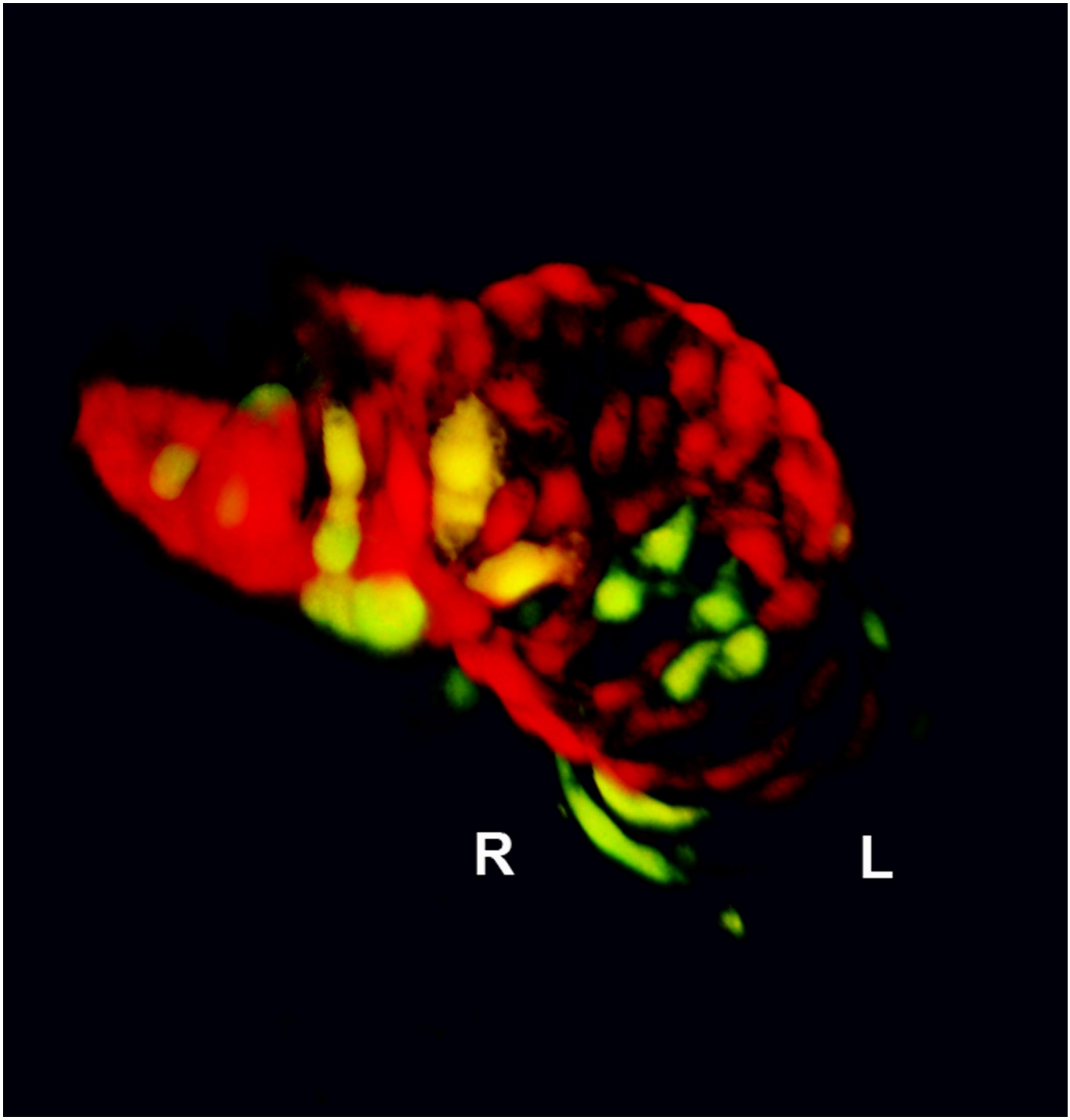
Movie S4. Original right myocardial cells of WT embryos become localized to the ventral side of the linear heart tube by 24 hpf. Dorsal view of a WT, left-jogged heart tube at 24–28 hpf, with ventricle positioned to the top and atrium to the bottom, of a *Tg*(*cmlc2:dsredt4*) embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the original right cardiac cells. A 360° rotation of the heart tube about the L/R axis reveals the restriction of these GFP-expressing cells to the ventral region of the linear heart.

Movie S4 (MOV)



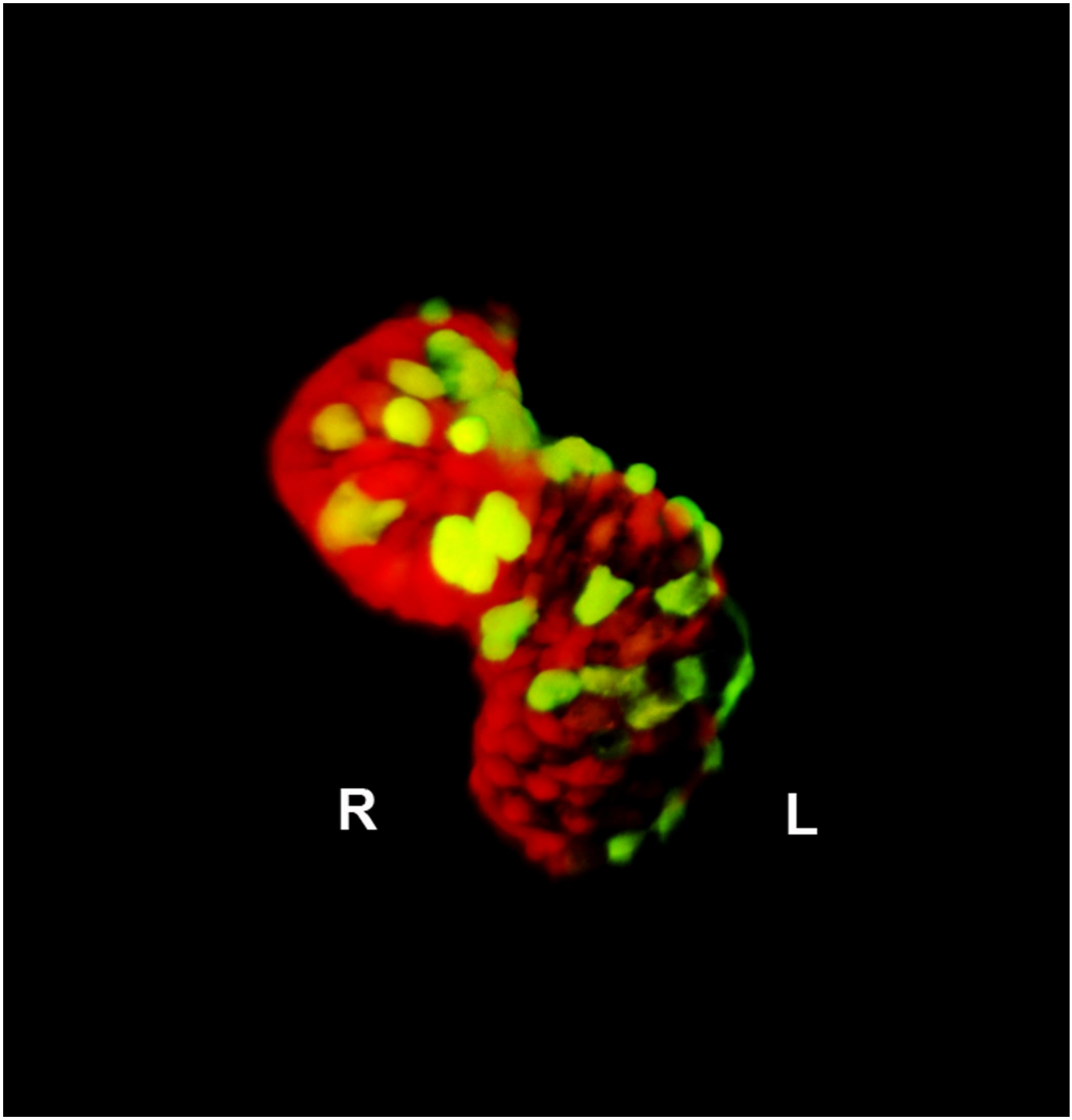
Movie S5. Original left myocardial cells of WT embryos are relocalized to the left side of the right-looped heart by 48 hpf. Ventral view of a WT, right-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) embryo with expression of *cmlc2::Dendra* GFP exclusively in the original left cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the left side of the looped heart and their contribution to the inner curvature of the ventricle and outer curvature of the atrium.

Movie S5 (MOV)



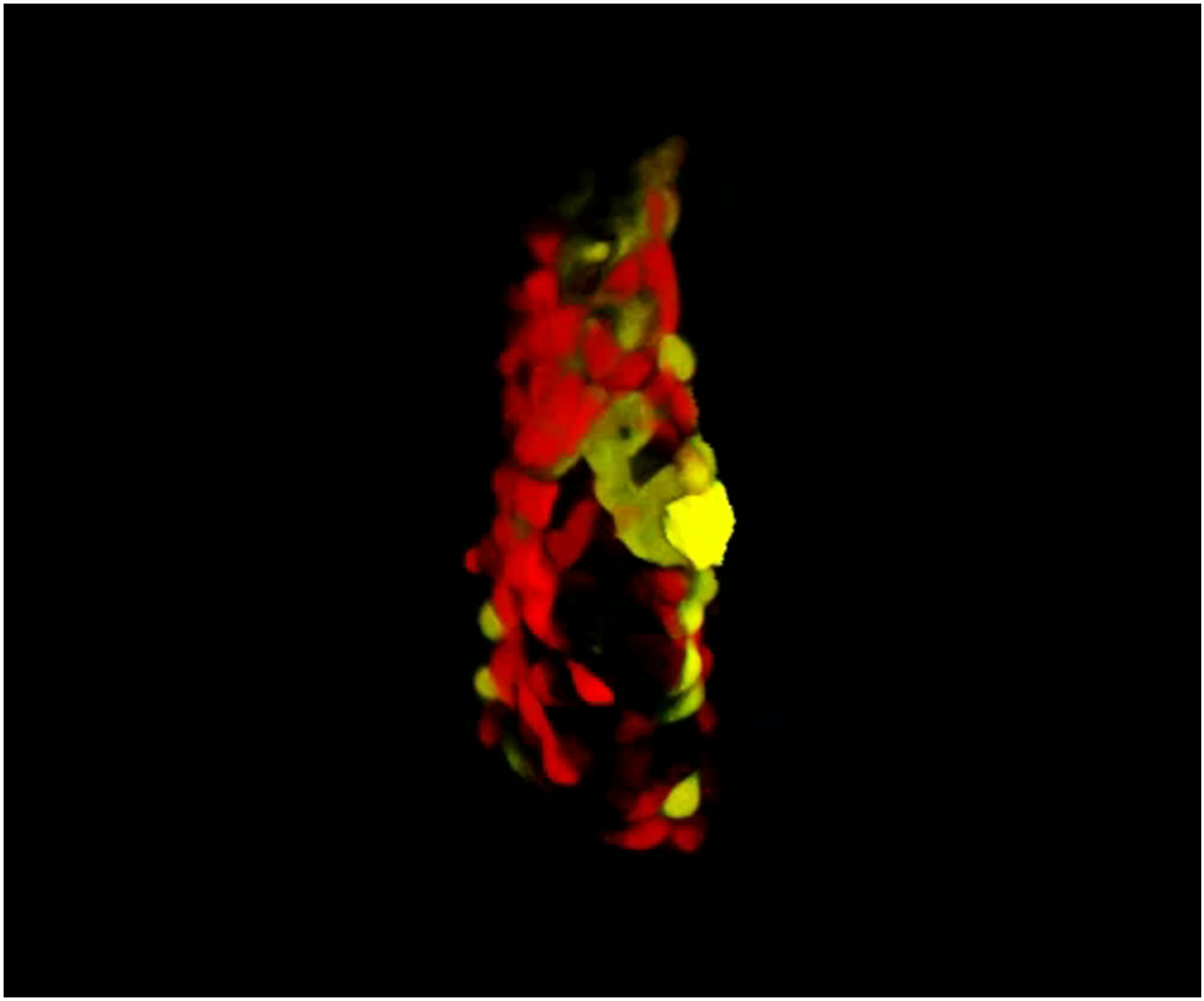
Movie S6. Original right myocardial cells of WT embryos are relocalized to the right side of the right-looped heart by 48 hpf. Ventral view of a WT, right-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the original right cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the right side of the looped heart and their contribution to the outer curvature of the ventricle and inner curvature of the atrium.

Movie S6 (MOV)



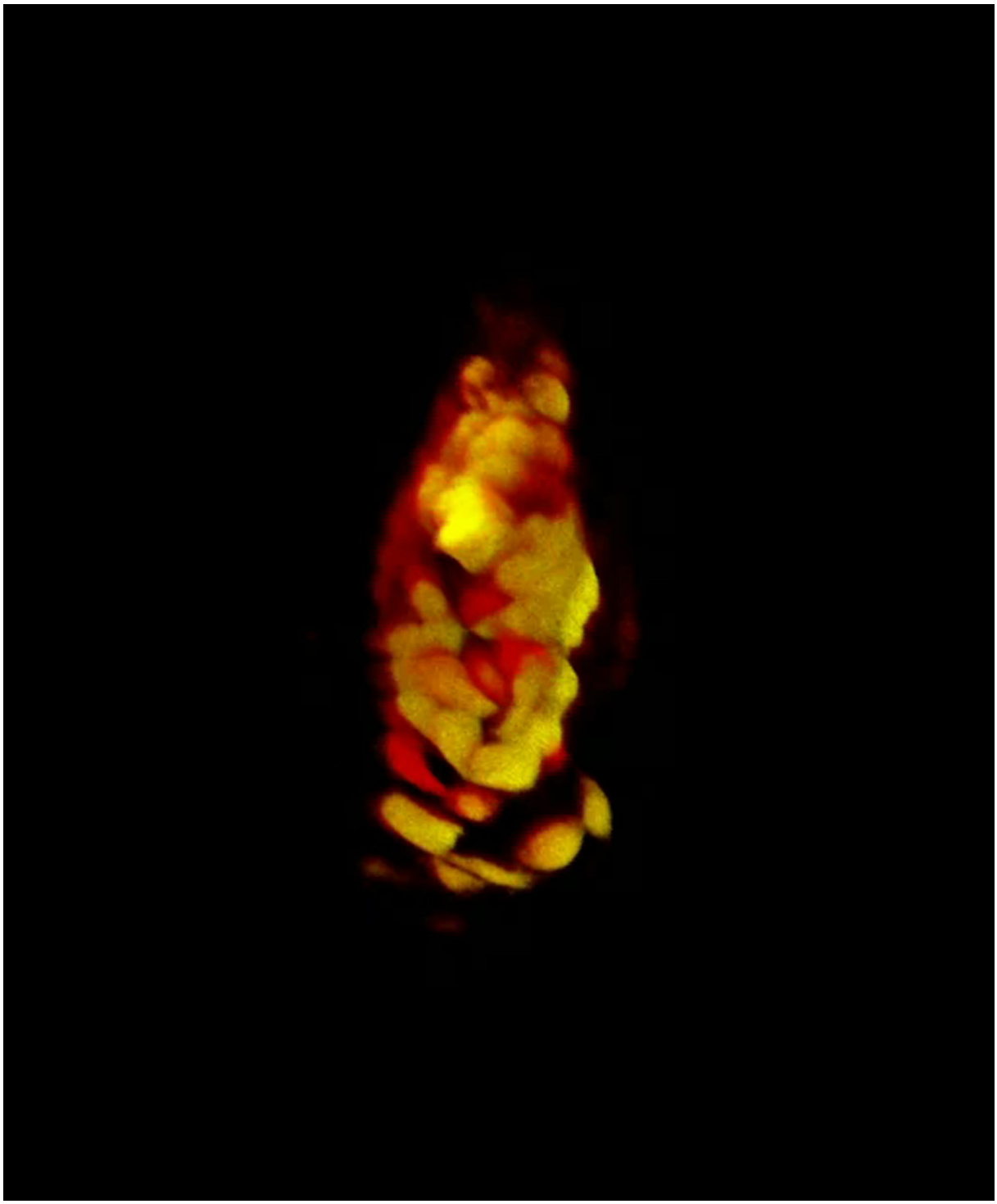
Movie S7. Original left myocardial cells of *swt* morphants with left cardiac jog are relocalized to the left side of the right-looped heart by 48 hpf. Ventral view of a right-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) *swt* morphant embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the original left cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the left side of the looped heart and their contribution to the inner curvature of the ventricle and outer curvature of the atrium.

Movie S7 (MOV)



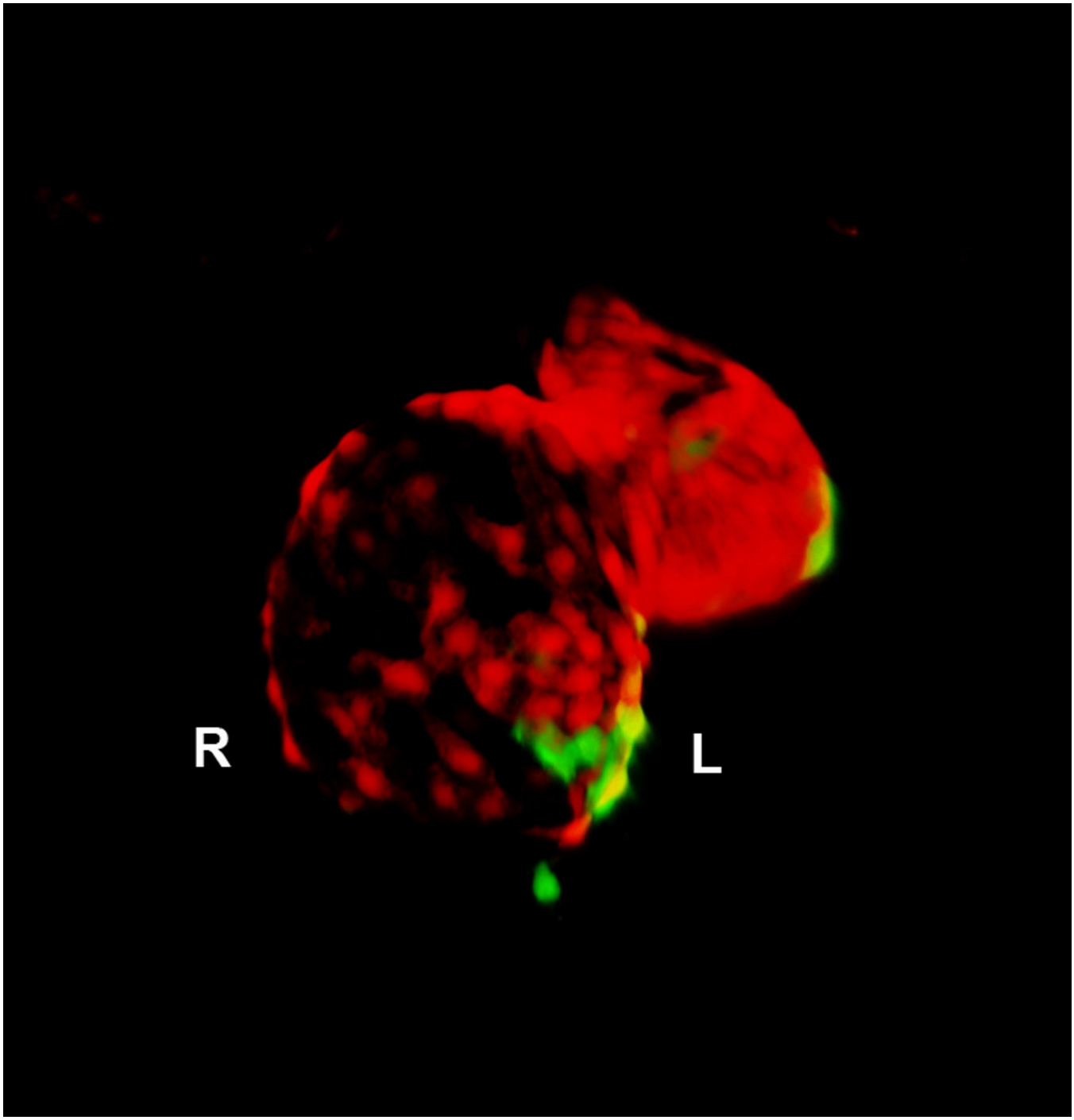
Movie S8. Original left myocardial cells of *swt* morphants with right jog become localized to the ventral side of the linear heart tube by 24 hpf. Dorsal view of the right-jogged heart tube at 24–28 hpf, with ventricle positioned to the top and atrium to the bottom, of a *Tg*(*cmlc2:dsredt4*) *swt* morphant with expression of *cmlc2*::*Dendra* GFP exclusively in the original left cardiac cells. A 360° rotation of the heart tube about the L/R axis reveals the restriction of these GFP-expressing cells to the ventral region of the linear heart.

Movie S8 (MOV)



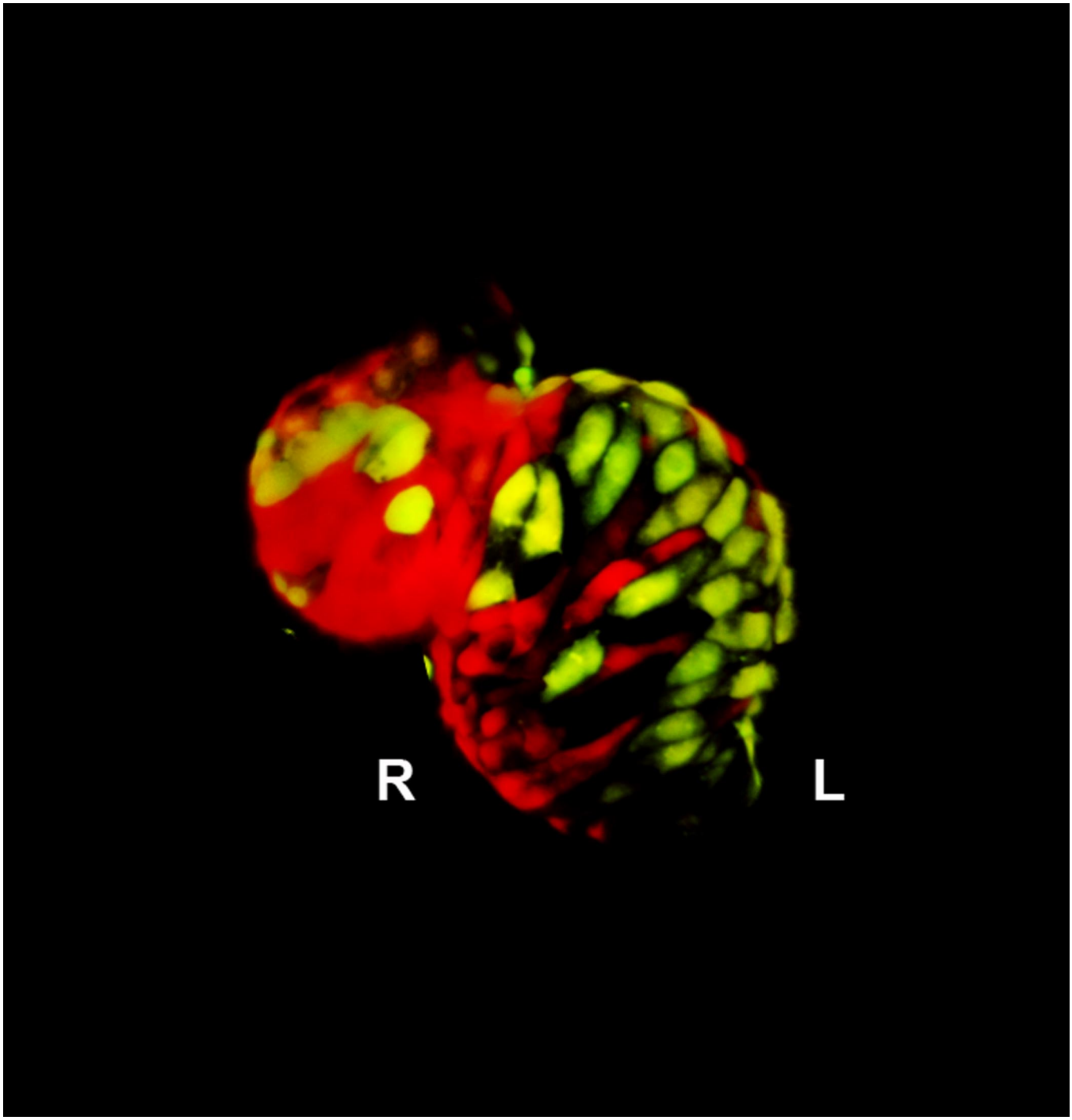
Movie S9. Original right myocardial cells of *swt* morphants with right jog become localized to the dorsal side of the linear heart tube by 24 hpf. Dorsal view of the right-jogged heart tube at 24–28 hpf, with ventricle positioned to the top and atrium to the bottom, of a *Tg*(*cmlc2:dsredt4*) *swt* morphant with expression of *cmlc2*::*Dendra* GFP exclusively in the original right cardiac cells. A 360° rotation of the heart tube about the L/R axis reveals the restriction of these GFP-expressing cells to the dorsal region of the linear heart.

Movie S9 (MOV)



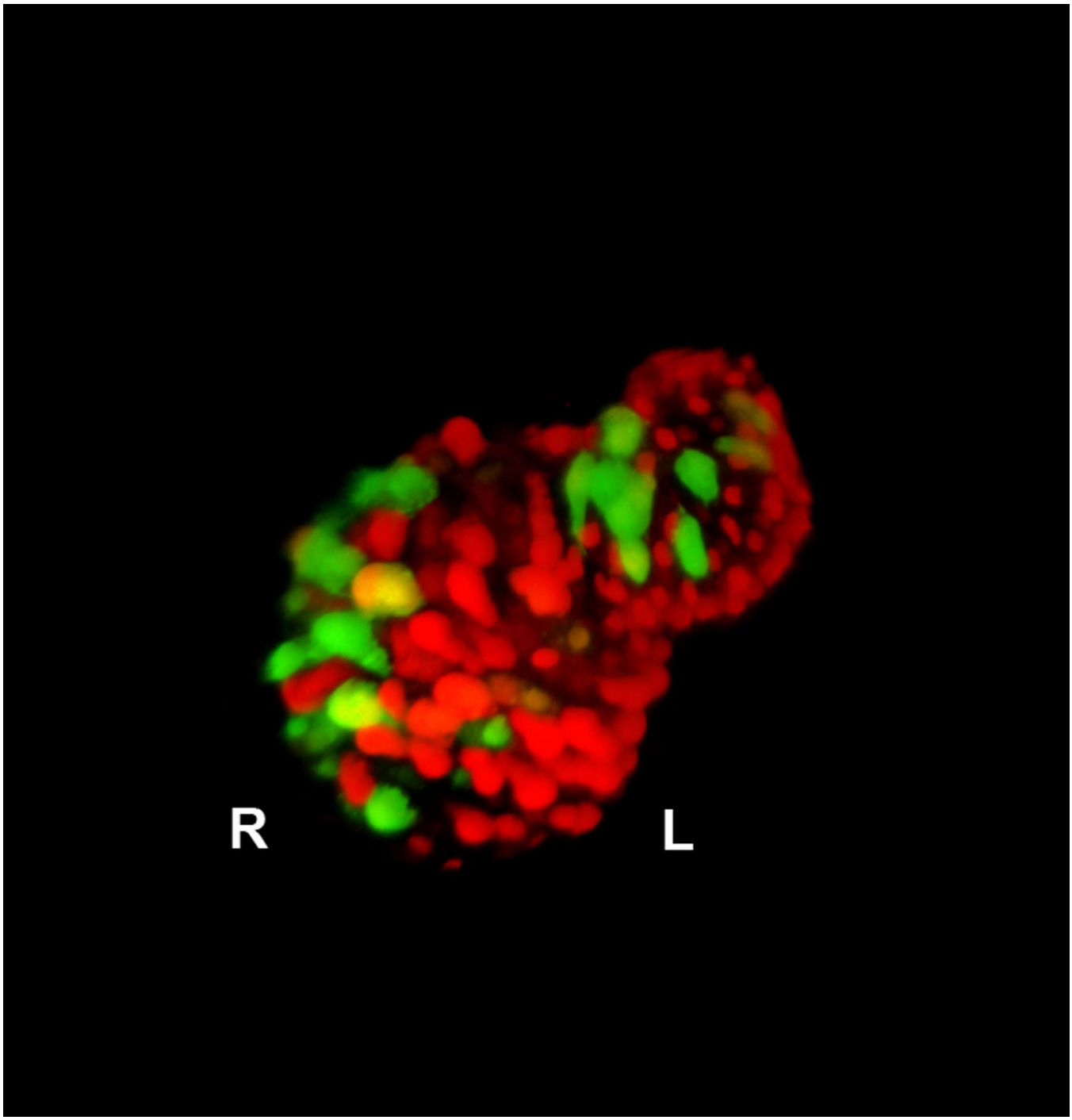
Movie S10. Original left myocardial cells of *swt* morphants with right cardiac jog are relocalized to the left side of the left-looped heart by 48 hpf. Ventral view of a left-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) *swt* morphant embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the original left cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the left side of the looped heart and their contribution to the outer curvature of the ventricle and inner curvature of the atrium.

Movie S10 (MOV)



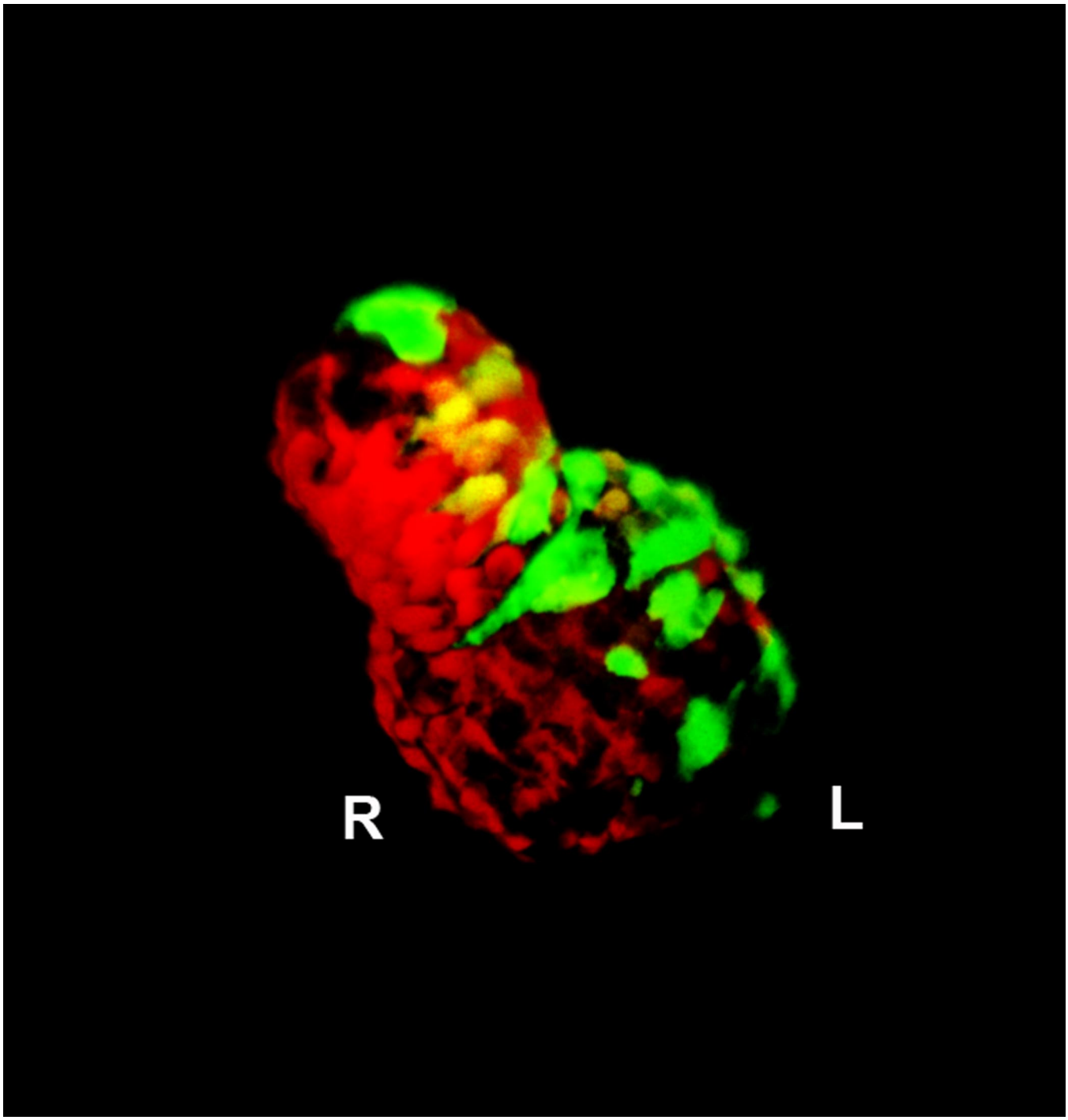
Movie S11. Original left myocardial cells of *spaw* morphants with left cardiac jog are relocalized to the left side of the right-looped heart by 48 hpf. Ventral view of a right-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) *spaw* morphant embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the original left cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the left side of the looped heart and their contribution to the inner curvature of the ventricle and outer curvature of the atrium.

Movie S11 (MOV)



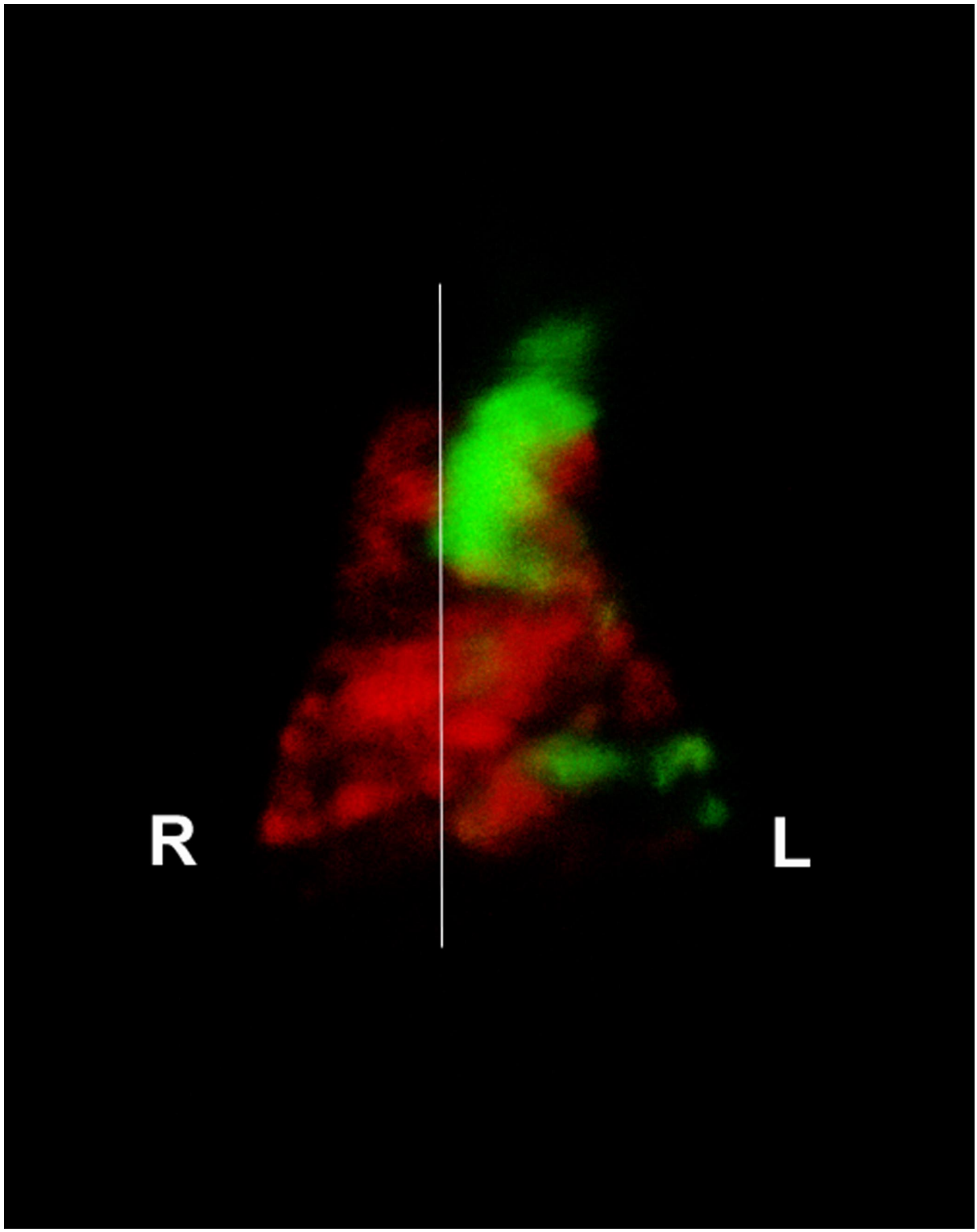
Movie S12. Original right myocardial cells of *spaw* morphants with right cardiac jog are relocalized to the right side of the left-looped heart by 48 hpf. Ventral view of a left-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) *spaw* morphant embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the right cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the right side of the looped heart and their contribution to the inner curvature of the ventricle and outer curvature of the atrium.

Movie S12 (MOV)



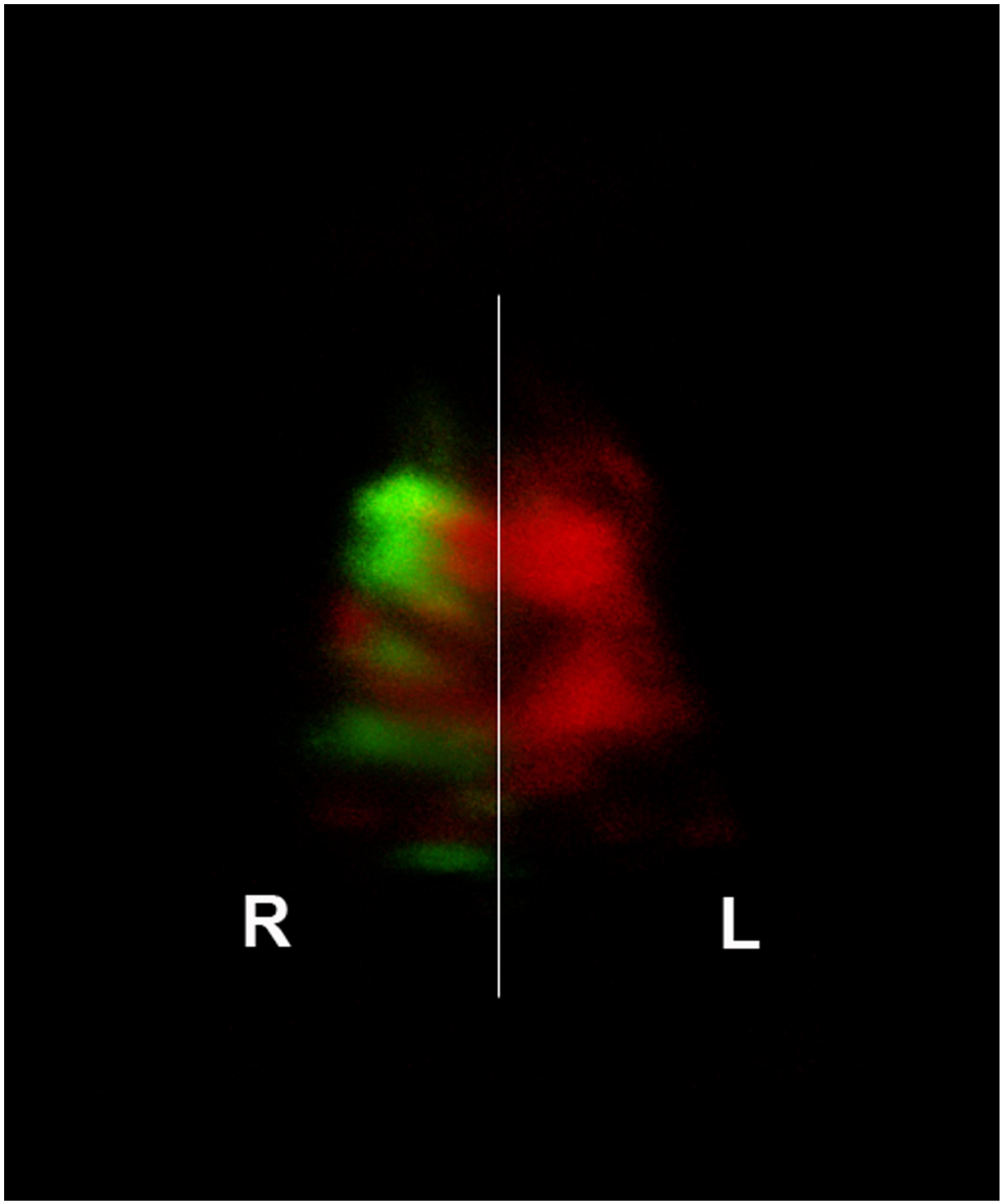
Movie S13. Original left myocardial cells of *spaw* morphants with right cardiac jog are relocalized to the left side of the right-looped heart by 48 hpf. Ventral view of a right-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) *spaw* morphant embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the left cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the restriction of these GFP-expressing cells to the left side of the looped heart and their contribution to the inner curvature of the ventricle and outer curvature of the atrium.

Movie S13 (MOV)



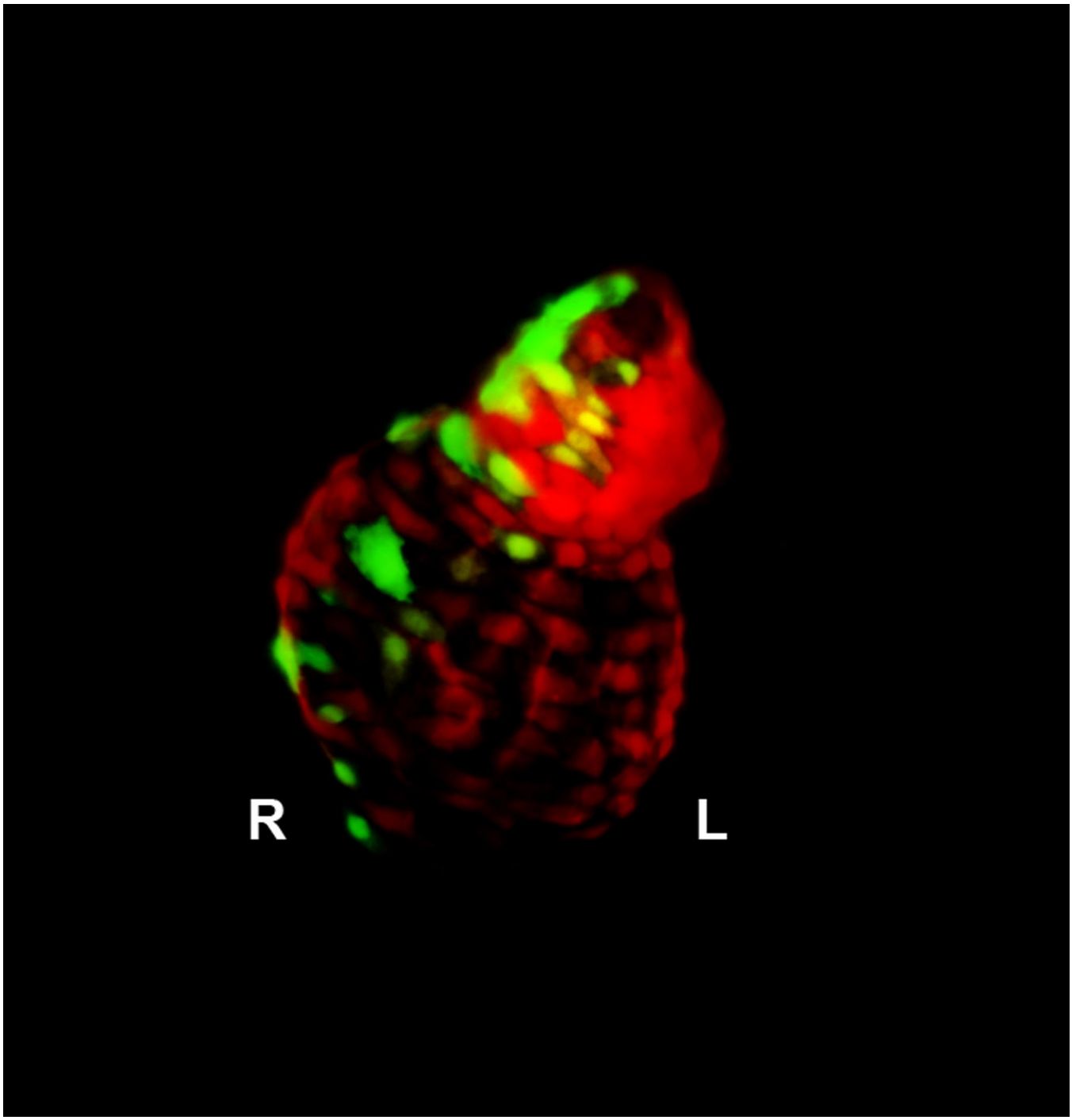
Movie S14. Original left myocardial cells of *spaw* morphants lacking directional jog are maintained on the left side of the linear heart tube at 24 hpf. Dorsal view of a no-jogged heart at 24–28 hpf, with ventricle positioned to the top and atrium to the bottom, of a *Tg*(*cmlc2:dsredt4*) *spaw* morphant with expression of *cmlc2*::*Dendra* GFP exclusively in the original left cardiac cells. Dotted white line represents the midline of the linear heart tube. A 360° rotation of the heart about the L/R axis reveals the maintenance of these GFP-expressing cells on the left side of the linear heart tube.

Movie S14 (MOV)



Movie S15. Original right myocardial cells of *spaw* morphants lacking directional jog are maintained on the right side of the linear heart tube at 24 hpf. Dorsal view of a no-jogged heart at 24–28 hpf, with ventricle positioned to the top and atrium to the bottom, of a *Tg*(*cmlc2:dsredt4*) *spaw* morphant with expression of *cmlc2*::*Dendra* GFP exclusively in the original right cardiac cells. Dotted white line represents the midline of the linear heart tube. A 360° rotation of the heart about the L/R axis reveals the maintenance of these GFP-expressing cells on the right side of the linear heart tube.

Movie S15 (MOV)



Movie S16. Original right myocardial cells of *spaw* morphants lacking directional jog are maintained on the right side of the heart through 48 hpf. Ventral view of a left-looped heart at 48 hpf in a *Tg*(*cmlc2:dsredt4*) *spaw* morphant embryo with expression of *cmlc2*::*Dendra* GFP exclusively in the right cardiac cells. Right and left sides of the embryo are indicated. A 360° rotation of the heart about the L/R axis reveals the maintenance of these GFP-expressing cells on the right side of the looped heart and their contribution to the inner curvature of the ventricle and outer curvature of the atrium.

Movie S16 (MOV)

Table S1. Alterations in asymmetric gene expression in *swt* and *spaw* morphant embryos

		Phenotypes of asymmetric gene expression, %					
Genotype	*n*	L *spaw* L *lefty2*	R *spaw* R *lefty2*	B *spaw* B *lefty2*	B *spaw* L *lefty2*	B *spaw* R *lefty2*	A *spaw* A *lefty2*
Wild type	38	97	0	0	3	0	0
swt morphant (9 ng)	68	32	38	4	11	15	0
spaw morphant (1 ng)	69	1	0	0	0	0	99

Gene expression data was obtained from embryos staged at 20–22 somites. L, left; R, right; B, bilateral; A, absent.

Table S2. Defects in and correlation between the directions of cardiac jogging and looping in *swt* and *spaw* morphant embryos

Genotype	*n*	Jogging, %	Right loop, %	Left loop, %	No loop, %
Wild type	183				
Left jog		98	99	1	0
Right jog		1	0	100	0
No jog		1	50	50	0
swt morphant (9 ng)	209				
Left jog		45	99	1	
Right jog		37	6	91	3
No jog		18	66	27	7
spaw morphant (1 ng)	465				
Left jog		38	89	9	2
Right jog		33	54	44	2
No jog		29	60	18	21

Jogging and looping directionality were scored in live embryos using light microscopy at 24–26 hpf and 48 hpf, respectively.

Table S3. Fate mapping of left and right cardiac fields

Genotype	*n*	Jogging, *n*	Right loop, *n*	Left loop, *n*	No loop, *n*
Wild type	22				
Left jog		22	22	0	0
Right jog		0	—	—	—
No jog		0	—	—	—
swt morphant (9 ng)	44				
Left jog		17	17	0	0
Right jog		26	0	26	0
No jog		1	0	1	0
spaw morphant (1 ng)	42				
Left jog		19	17	2	0
Right jog		11	6	5	0
No jog		12	8	4	0

All embryos were individually followed through development and exhibited left and right GFP localization as described in the results. Jogging and looping direction were scored in live embryos using light microscopy at 24–26 hpf and 48 hpf, respectively. *n* values rather than percentages are provided as these data are meant to display the total number of embryos analyzed in these experiments. The artificial selection placed on these clutches for embryos exhibiting left or right-restricted GFP prevents an accurate determination of percentage for the overall jogging phenotypes observed in *swt* or *spaw* morphants. These numbers and percentages are provided in Table S2.