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Transcriptome analysis of regeneration during *Xenopus laevis* experimental twinning

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ABSTRACT Animal embryos have the remarkable property of self-organization. Over 125 years ago, Hans Driesch separated the two blastomeres of sea urchin embryos and obtained twins, in what was the foundation of experimental embryology. Since then, embryonic twinning has been obtained experimentally in many animals. In a recent study, we developed bisection methods that generate identical twins reliably from *Xenopus* blastula embryos. In the present study, we have investigated the transcriptome of regenerating half-embryos after sagittal and dorsal-ventral (D-V) bisections. Individual embryos were operated at midblastula (stage 8) with an eyelash hair and cultured until early gastrula (stage 10.5) or late gastrula (stage 12) and the transcriptome of both halves were analyzed by RNA-seq. Since many genes are activated by wound healing in *Xenopus* embryos, we resorted to stringent sequence analyses and identified genes up-regulated in identical twins but not in either dorsal or ventral fragments. At early gastrula, cell division-related transcripts such as histones were elevated, whereas at late gastrula, pluripotency genes (such as *sox2*) and germ layer determination genes (such as *eomesodermin, ripply2* and *activin receptor ACVRI)* were identified. Among the down-regulated transcripts, *sizzled*, a regulator of Chordin stability, was prominent. These findings are consistent with a model in which cell division is required to heal damage, while maintaining pluripotency to allow formation of the organizer with a displacement of 90^0 from its original site. The extensive transcriptomic data presented here provides a valuable resource for data mining of gene expression during early vertebrate development.

KEY WORDS: *Xenopus laevis, twinning, regeneration, pluripotency, self-organization*

Self-organization is a remarkable property of animal embryos. Experimental embryology started when Hans Driesch separated the first two blastomeres of sea urchin embryos and obtained twin larvae (Driesch, 1891). In insects, duplicated embryos can be obtained by partially sectioning the blastoderm (Krause and Sander, 1962). In the chick, when the early blastoderm is sliced into four fragments multiple embryos are formed (Spratt and Haas, 1960), and removal of the hypoblast or inhibition of Nodal signaling also leads to the formation of multiple primitive streaks (Bertocchini and Stern, 2002). In humans, monozygotic twins are delivered in three of 1,000 live births (Hall, 2003), and the armadillo has obligate polyembryony giving rise to identical quadruplets from each egg (Carter, 2018). Mammalian embryonic stem cells in culture have the ability to self-organize into complex organoids (Sasai, 2013). Recently, gastrula-like aggregates have been obtained by self-assembly of mouse (Sozen *et al.*, 2018) or human (Warmflash *et al.*, 2014; Martyn *et al.*, 2018) embryonic stem cells under appropriate conditions.

In Amphibia, the study of twinning has a long history. When one of the two initial blastomeres of the frog embryo is removed,

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a complete embryo can result (Morgan, 1895). This represents the ultimate feat in regeneration, for the missing half of the body, with all the tissues that this implies, can regenerate from only half an egg. Using partial constriction with a baby hair loop, Spemann obtained two well-proportioned embryos from the same salamander egg (Spemann, 1901). In *Xenopus*, identical twins can be generated after sagittally cutting the embryo with a scalpel (De Robertis, 2006) but at very low frequencies, precluding meaningful mechanistic studies. However, in a recent study we improved methods for obtaining *Xenopus laevis* identical twins by using an eyelash knife and improved culture conditions (Moriyama and De Robertis, 2018). Using cell lineage studies we could recently

analyze the developmental mechanism of twinning. We found that the large wound caused by sagittal bisection closes rapidly from all directions within an hour, so that tissue that normally would have been the most dorsal becomes directly juxtaposed to ventral-most cells at the healing point. Formation of the future dorsal lip (*i.e.*, the Spemann organizer) was displaced by 90° from the healing point. The BMP (Bone Morphogenetic Protein) gradient marked by nuclear phospo-Smad1/5/8 was also displaced, but in the opposite direction. In this way, the entire D-V morphogenetic gradient is respecified by repositioning signaling centers (Moriyama and De Robertis, 2018).

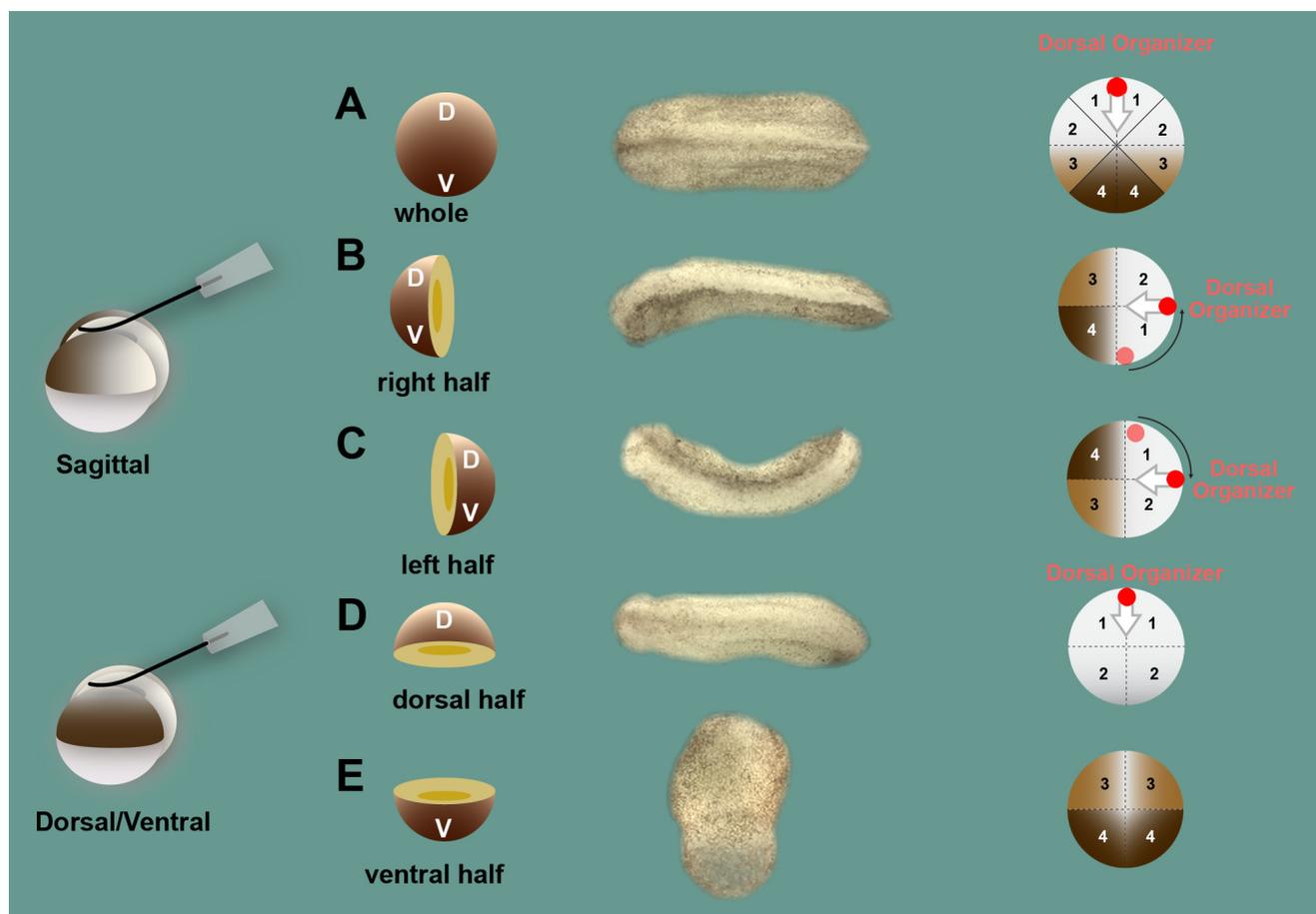The recent completion of the *Xenopus laevis* genome sequence

Fig. 1. Diagram of the experimental procedure used to investigate regeneration in *Xenopus laevis* half embryos. *On the left, drawing of blastula embryos being bisected in the sagittal or Dorsal/Ventral directions. Middle, the five samples used to generate RNA-seq libraries.* **(A)** *Uncut whole embryo.* **(B)** *Right half embryo cut sagittally; note that the maternal pigment is darker on the left side of the tailbud tadpole (while it is uniform in the whole embryo).* **(C)** *Left half from the same blastula; note that pigment asymmetry is concentrated on the right side.* **(D)** *Dorsal half embryo at tailbud showing almost perfect scaling of the half embryo along the antero-posterior axis.* **(E)** *Ventral half embryo; because it lacks the Spemann organizer it develops into a belly-piece consisting of ventral tissues, such as blood and lateral plate, without any dorsal axis (Spemann, 1938). Photographs for right and left half tailbud embryos are reproduced, with permission, from Moriyama and De Robertis, 2018, while dorsal and ventral half embryos are unpublished but from the same experimental batch. On the right-hand side, diagrams showing how the displacement by 90° of the Spemann organizer explains the pigment asymmetry observed in twinned embryos (Moriyama and De Robertis, 2018). Numbers indicate the four segments into which the 16-cell embryo can be lineage-traced. After bisection the dorsal-most segment 1 becomes juxtaposed (at the wound healing point) to the ventral-most segment 4, which has high BMP and Wnt expression potential. The organizer is not yet formed at midblastula, but at early gastrula the new dorsal lip (organizer indicated by the red dot) forms 90° away from its original D-V location. Embryos in which both halves heal within 60 minutes and in which the organizer forms 90° away from the healing point have a high percentage (around 80%) of correct twinning. Segments 4 and 3 form the epidermis, and involution (white arrow) of the displaced organizer splits them into a less pigmented and more pigmented half, thus explaining the intriguing pigmentation asymmetry observed (Moriyama and De Robertis, 2018). Note that in the dorsal half embryo pigmentation is symmetric. Since individual embryos from this five conditions were sequenced (10^7 100 bp reads) in triplicate at early and late gastrula, the data for 16,000 gene transcripts presented in the searchable Excel Tables S1 and S2 represents a treasure trove of gene expression at the gastrula stage.*

TABLE 1

TRANSCRIPTS INDUCED BY SAGITTAL BISECTION IN STAGE 10.5 EMBRYOS*

	JGI9 ID	Human ID	Con.	L. half	R. half	D. half	V. half	p-value	Exp. #
Dorsal	Xelaev18001465m	HIST2H3A	4.02	22.89	17.11	7.34	1.70	0.15	1
	Xelaev18002543m	HIST2H3A	6.96	18.03	17.58	13.83	4.34	0.03	2
	Xetrov90022513m.1	HISTH4	1.57	8.34	8.01	5.73	1.99	0.03	1
	Xelaev18026398m	HISTH4	1.69	6.44	4.46	4.22	0.83	0.17	2
Ventral	krt12.S	KRT12	23.40	46.94	63.96	4.45	22.45	0.42	3
	krt.L	KRT19	1.82	25.71	17.90	0.44	3.09	0.21	3
	Xetrov90029514m.1	HISTH3	3.35	11.66	18.44	3.18	6.57	0.08	3
	Xetrov90003601m.S	#N/A	2.26	16.10	11.04	2.15	9.98	0.45	2
	sp7.L	SP7	1.41	4.80	5.30	1.34	3.40	0.07	3

*Listed according to average RPKM levels in left and right half-embryos.

by Harland and colleagues opened important experimental possibilities to study embryogenesis in this classical vertebrate model system (Session *et al.*, 2016; Briggs *et al.*, 2018). For example, the gene targets of the late Wnt signal (Kjolby and Harland, 2016) and of the early Wnt signature (Ding *et al.*, 2017a) have been elucidated in detail. Three new Wnt antagonists expressed in the Spemann organizer – Pkdcc/Vlk, Angptl4 and Bighead - were identified in our laboratory using these sensitive techniques (Ding *et al.*, 2017b; Kirsch *et al.*, 2017; Ding *et al.*, 2018).

Hoping to identify regeneration master control genes during twinning, we performed RNA-seq analysis on sagittal bisections of *Xenopus* embryos. Our initial study (Moriyama and De Robertis, 2018) did not reveal any significant reproducible changes in up-regulated transcripts in triplicate samples, and we speculated this could be due to the possibility that the organizer displacement might not be accompanied by changes in overall gene expression, or to our use of pools of 6 half-embryos per library with some variability in the orientation of the sagittal cut (Moriyama and De Robertis, 2018).

In the present study, we analyzed the transcriptomes of regenerating *individual* half-embryos. The amount of RNA was sufficient for generating RNA-seq libraries yielding 10^9 base pairs each. Stringent analytical conditions were necessary in order to exclude wound-healing genes that are activated by cutting independently of the orientation of the bisection. Sagittal left and right halves were compared to dorsal and ventral bisections. This allowed us to detect genes that were specifically increased in twinning embryos bisected sagittally at midblastula and cultured until early or late gastrula, but not in D-V half embryos. A few up-regulated genes were identified, which were involved in stem cell and germ layer specification. Among the down-regulated genes *sizzled*, a regulator of Chordin stability, was identified.

Results

Fig. 1 illustrates the experimental procedure. *Xenopus* embryos are a very favorable material for these investigations because the dorsal side can be identified by a less pigmented dorsal crescent from 2-cell onwards due to the rotation of the egg cortex. Regularly cleaving embryos were selected at 4-cell stage (Klein, 1987) and then bisected using an eyelash knife at mid-blastula (stage 8) (Nieuwkoop and Faber, 1967) following the sagittal or D-V axis perpendicular to it (Fig. 1, left) in 0.3 x modified Barth's solution (MBS; Gurdon, 1976). After one hour, embryos were transferred to 0.1 x MBS and cultured until early gastrula stage 10.5 or late

gastrula stage 12. When siblings were allowed to develop until tailbud (stage 22), complete regeneration could be seen. Fig. 1 shows images for (A) whole embryos, (B) right half embryos, (C) left half embryos, (D) dorsal half embryos (displaying a very complete scaling of pattern) and (E) ventral half embryos (that lack an organizer and all axial structures). The diagrams on the right-hand side of Fig. 1 depict the interpretation of the displacement of Spemann organizer that explains the developmental mechanism of twinning (Moriyama and De Robertis, 2018).

RNA-seq libraries were generated in triplicate for whole control, left sagittal, right sagittal, dorsal and ventral regenerating half-embryos at early and late gastrula. This meant a total of 30 RNA-seq libraries each one generating about 10^7 100-bp single-end reads. All embryos were from the same clutch. These data are presented as RPKM (Reads per Kilobase per Million mapped reads) in Supplementary Table S1 for stage 10.5, and Supplementary Table S2 for stage 12. This information represents a powerful resource for vertebrate embryology, not just for the twinning of sagittal halves but also for D-V regulation of gene expression.

A factor that severely complicates embryonic RNA-seq analyses in *Xenopus* is the wound-healing response of the embryo. We have described a signature of 743 genes that are up-regulated simply by embryo dissection (Ding *et al.*, 2017b). To circumvent this problem, we took a very stringent approach. Only genes that were increased by more than two-fold with respect to a corresponding uncut embryo were selected (including a difference of 2 RPKM to eliminate genes expressed at low levels). Since injury response genes are similarly activated in both dorsal and ventral fragments, we then chose only genes that were enriched in either the dorsal or ventral fragments. While these multiple selections likely eliminate many relevant genes that are expressed even a fraction more in dorsal and ventral fragments than in sagittal bisections, such an approach gave us the opportunity of detecting transcripts that were activated specifically in regenerating twins, which we had failed to find in previous experiments using pooled half embryos (Moriyama and De Robertis, 2018). Since supplementary Tables S1 and S2 are genome-wide, any transcriptional changes not highlighted here will be represented in this open-source data.

Transcripts up-regulated in twinning embryos

Table 1 shows the transcripts elevated in sagittal halves at stage 10.5. From the bisection at midblastula to RNA preparation at gastrula about 4.5 hours elapsed, including many injury-response genes. Only 9 genes were scored as elevated in sagittally bisected embryos under the stringent conditions described above. Of these,

TABLE 2

TRANSCRIPTS INDUCED BY SAGITTAL BISECTION IN STAGE 12 EMBRYOS*

	JGI9 ID	Human ID	Con.	L. half	R. half	D. half	V. half	p-value	Exp. #
Dorsal	Xelaev18001880m	SOX2	12.52	31.11	32.93	28.52	1.64	0.45	1
	eomes.S	EOMES	9.88	24.37	23.90	23.39	3.28	0.05	1
	apoc1-like.L	APOC1	5.57	13.08	24.48	11.28	3.85	0.04	1
	fkbp9.L	FKBP9	8.49	18.13	17.49	14.63	7.27	0.01	1
	Xelaev18016415m	SERPINH1	1.35	15.08	10.96	8.23	2.89	0.10	3
	Xelaev18016415m	SERPINH1	2.39	13.25	11.15	5.06	0.85	0.10	1
	LOC100487498.L	RIPPLY2	3.67	7.96	13.33	4.72	0.23	0.37	1
	LOC100487498.S	RIPPLY2	3.73	9.63	10.52	6.04	0.62	0.37	1
	Xelaev18029489m	ZNF451	3.13	9.09	9.95	7.72	3.68	0.31	1
	Xelaev18003368m	#N/A	3.03	6.42	6.32	6.15	1.68	0.03	1
	acvr1.L	ACVR1	2.93	6.13	5.89	5.69	2.31	0.04	1
	Xelaev18036787m	CISD1	0.72	4.18	6.31	3.10	2.89	0.11	3
	LOC108701590	FAM220A	0.00	4.22	4.46	3.64	0.83	0.25	3
	Xetrov90002007m.1	NR2C2AP	0.00	3.11	4.09	2.24	0.00	0.32	1
Ventral	Xetrov90029514m.1	HISTH3	7.14	23.94	16.74	2.69	9.87	0.02	3
	fucolectin-5-like	#N/A	5.16	16.37	23.89	1.31	4.29	0.11	1
	fucolectin.L	#N/A	2.15	20.47	16.81	1.23	6.25	0.06	1
	comt.L	#N/A	2.61	15.69	17.65	2.61	8.68	0.28	3
	Xelaev18025569m	EP300	6.06	14.23	15.42	4.60	11.73	0.27	1
	Xelaev18002343m	HIST2H2AB	2.60	10.12	8.57	3.27	7.50	0.48	3
	Xelaev18039781m	SGPP1	4.17	8.81	8.68	1.90	8.30	0.42	1
	LOC101731107.L	MIEN1	1.38	10.03	6.79	0.86	6.34	0.32	3
	Xelaev18037854m	GPR161	2.95	6.24	6.40	1.40	6.13	0.11	1
	Xelaev18037313m	ST3GAL4	1.42	4.82	5.27	0.29	2.44	0.09	1
	tpx2.S	TPX2	0.58	3.83	5.04	1.10	3.36	0.39	3

*Listed according to average RPKM levels in left and right regenerating half-embryos.

5 were histones, which were reproducible between individual embryos analyzed in the 3 experiments; a possible explanation is an increase in cell division or chromatin reorganization during twinning. In addition, two epidermal keratins, *KRT12* and *KRT19* were found (Gawantka, *et al.*, 1998) in ventrally enriched transcripts. The transcription factor *SP7* (also known as *Osterix*), a well-characterized target of Wnt signaling in *Xenopus* development (Kjolby and Harland, 2016; Nakamura *et al.*, 2016) was also increased. Xenbase.com provides a rich resource for *in situ* expression pattern of *SP7* (the neural ridge at neurula), and for that of many of the other genes identified in these genome-wide analyses.

The results at late gastrula are shown in the heatmap of Fig. 2 and in Table 2. Embryos were cut at midblastula and allowed to regenerate for about 9 hours at stage 12. In one of the three experiments, one of the sagittal halves was ventralized and the other dorsalized, indicating that in this particular embryo the bisection missed the sagittal plane (experiment 2 in Table S2). The other two experiments showed no D-V preference, indicating that the midline was properly bisected (experiments 1 and 3 in Table S2). A total of 25 genes were up-regulated preferentially in regenerating sagittally cut twins.

Interestingly, when ranked according to RPKM levels, the top gene increased by sagittal bisection was *SOX2* (Table S2), a gene essential for the pluripotent and self-renewing properties of embryonic stem cells (Takahashi and Yamanaka, 2006), and an early dorsal gastrula and later a neural plate marker in *Xenopus* (see Xenbase and De Robertis *et al.*, 1997). The next gene was *EOMES*, a T-box transcription factor crucial for endomesodermal differentiation in *Xenopus* and mouse (Ryan *et al.,* 1996; Russ *et al.*, 2000) expressed strongly in dorsal mesoderm and more weakly

in ventral mesoderm at gastrula (see Xenbase). The transcription of *eomesodermin* is regulated by *Sox2*, which together with *Oct4* and *Nanog* binds to the core regulatory circuitry in human embryonic stem cells (Boyer *et al.*, 2011; Teo *et al.*, 2011). *SERPINH1* transcripts were increased in sagittal sections of both experiments (Table 2). This is a protein required for the proper folding of the triple helix of Procollagen I, which when mutated causes osteogenesis imperfecta (Ito and Nagata, 2017). Two other genes, *RIPPLY2,* and *FUCOLECTIN* were up-regulated in two alloalleles in regenerating twins but within the same experiment (Table 2). *Xenopus laevis* is an allotetraploid species and many genes are present in two copies in Long or Short chromosomes (Session *et al.*, 2016). When mutated, *RIPPLY2* causes vertebral segmentation defects in humans (McInerney-Leo *et al.*, 2015). Interestingly, RIPPLY2 is a regulator of T-box proteins (the family to which Eomesodermin belongs), converting Tbx6, Tbx24 and Brachyury from activators to repressors. This is achieved by recruiting the transcriptional repressor Groucho 4 (Kawamura *et al.*, 2008) and by recruiting Tbx6 to the proteasome for degradation (Zhao *et al.*, 2018). *Xenopus laevis ripply2* is expressed in presomitic mesoderm (according to Xenbase). FUCOLETIN (also known in *Xenopus* as epilectin) is a lectin that binds fucose and is induced by Bone Morphogenetic Proteins (BMP) in ventral ectoderm at stage 12 (Massé *et al.*, 2004; Xenbase).

Other genes induced in sagittal regenerating embryos were found only once (Table 2). *APOC1*, encodes Apolipoprotein C1, a protein that binds to very low density lipoproteins in serum and has the interest of being a Wnt-induced gene required for neural crest formation (Yokota *et al.*, 2017). *FKBP9* is a peptidyl-prolyl cis-trans isomerase (Jo *et al.*, 2001). *ZNF451* is an epidermal gene

TABLE 3

TRANSCRIPTS REPRESSED BY SAGITTAL BISECTION IN STAGE 10.5 EMBRYOS*

	JGI9 ID	Human ID	Con.	L. half	R. half	D. half	V. half	p-value	Exp. #
Ventral	szl.L	SFRP2	34.93	8.20	8.56	1.57	241.04	0.05	3
	Xelaev18000342m	#N/A	29.95	4.14	4.71	19.68	48.09	0.12	2
	szl.S	SFRP2	24.83	5.88	9.32	1.55	141.34	0.18	3
	Xelaev18000337m	ZCCHC3	16.09	3.84	3.87	24.34	51.12	0.03	1
	Xelaev18040512m	DAPK2	13.96	6.50	6.90	6.33	26.00	0.06	3
	Xelaev18000342m	#N/A	11.26	0.88	1.47	20.38	49.13	0.12	1
	degs3.L	DEGS2	8.57	3.46	3.19	3.48	12.72	0.02	3
	Xetrov90028501m.1	#N/A	6.17	1.19	2.12	2.62	28.45	0.14	3
	Xelaev18003951m	#N/A	5.37	0.00	0.62	1.19	20.26	0.15	1
	Xelaev18010379m	TBX3	4.31	1.54	2.02	0.94	11.16	0.05	2
	Xetrov90028501m.1	#N/A	3.72	0.61	0.58	4.65	11.06	0.14	1
	Xelaev18009910m	CRYGB	3.44	0.41	0.82	1.51	5.63	0.07	3
	Xelaev18034023m	#N/A	1.77	0.42	0.14	2.90	7.31	0.09	1
Dorsal	Xelaev18004073m	#N/A	15.03	1.19	0.00	21.40	7.09	0.05	3
	Xelaev18019555m	PLSCR3	2.09	0.50	0.00	6.45	0.77	0.14	2

*Listed according to average RPKM levels in control-embryos.

in *Xenopus* (Gawantka *et al.*, 1998). *ACVR1* is Activin A Receptor Type, a gene important for mesoderm induction (Asashima *et al.*, 2008). *CISD1* is an outer-mitochondrial membrane protein (Wiley *et al.*, 2007). *FAM220A* and *NR2C2AP* are little-known proteins. *COMT* is chatechol-O-mehtyltransferase-like. *EP300* is the well-known histone acetyl-transferase transcriptional coactivator p300. *SGPP1* is Sphingosine-1 phosphate phosphatase 1. *MIEN1* is the secreted protein migration and invasion enhancer 1 involved in cancer metastases. *GPR161* encodes a G protein-coupled 7 transmembrane receptor that has the interest of being important for Hedgehog signaling at the level of stereocilia (Hwang *et al.*, 2018). *ST3GAL4* is *b*-galactoside α-2,3-sialyltransferase 4, an enzyme that adds sialic acid to glycoproteins in the cell surface (Harduin-Lepers *et al.*, 2001). Finally, *TPX2* is a microtubule-associated spindle assembly factor.

Taken together, this whole-genome transcriptome data of genes up-regulated during twinning support a model in which cell division is required at early gastrula, while at late gastrula pluripotency and germ-layer specification genes show increased expression in identical twins.

Transcripts down-regulated in twinning embryos

Genes down-regulated by sagittal bisection were also analyzed. Table 3 displays 25 transcripts repressed in sagittally bisected embryos at stage 10.5 that were identified using very stringent conditions (two-fold repression in sagittal halves and over two-fold induction in ventral or dorsal fragments with respect to controls embryos) to eliminate wound response genes. When ranked according to difference between control and sagittal RPKM levels, the gene most repressed by sagittal bisection was *Sizzled* (Table 3), a competitive inhibitor of Xolloid proteinases and a major regulator of BMP signaling during gastrulation (Lee *et al.*, 2006). Sizzled is an sFRP (secreted frizzled-related protein, most related to human *SFRP2*) expressed in the ventral side of the gastrula (see Xenbase) and fulfills a critical role in D-V patterning by acting as a competitive inhibitor of Chordin degradation (Plouhinec *et al.*, 2013). Thus, when Sizzled levels are low, Chordin protein levels are expected to be high (De Robertis *et al.*, 2017). Sizzled was also the only gene found twice, both in its Long and Short forms present in the *Xenopus laevis* allotetraploid species.

Thirteen other transcripts were also decreased in sagittal half-

TABLE 4

TRANSCRIPTS REPRESSED BY SAGITTAL BISECTION IN STAGE 12 EMBRYOS*

	JGI9 ID	Human ID	Con.	L. half	R. half	D. half	V. half	p-value	Exp. #
Ventral	Xelaev18030475m	ZNF268	57.21	27.46	22.47	19.45	89.24	0.01	1
	acaa2.S	ACAA2	37.49	16.73	13.90	17.14	80.33	0.08	1
	Xelaev18040968m	ATP6V1A	34.71	12.63	14.50	19.36	80.57	0.05	1
	Xelaev18000342m	#N/A	32.77	7.11	15.06	18.18	88.12	0.00	3
	foxj1.S	FOXJ1	19.67	9.11	5.72	7.68	36.08	0.03	1
	foxi1.L	FOXI1	13.31	1.88	2.59	0.81	22.54	0.03	1
	Xelaev18008287m	MCIDAS	6.15	2.17	1.71	0.16	19.57	0.12	1
	cebpd.L	CEBPD	5.99	1.16	0.87	1.66	12.68	0.00	3
	capn8.S	CAPN8	5.58	2.35	1.92	1.62	20.03	0.00	3
	Xelaev18008287m	MCIDAS	4.41	1.61	0.91	0.92	10.91	0.12	3
	Xelaev18043178m	UBP1	4.06	1.48	0.50	0.64	13.20	0.28	3

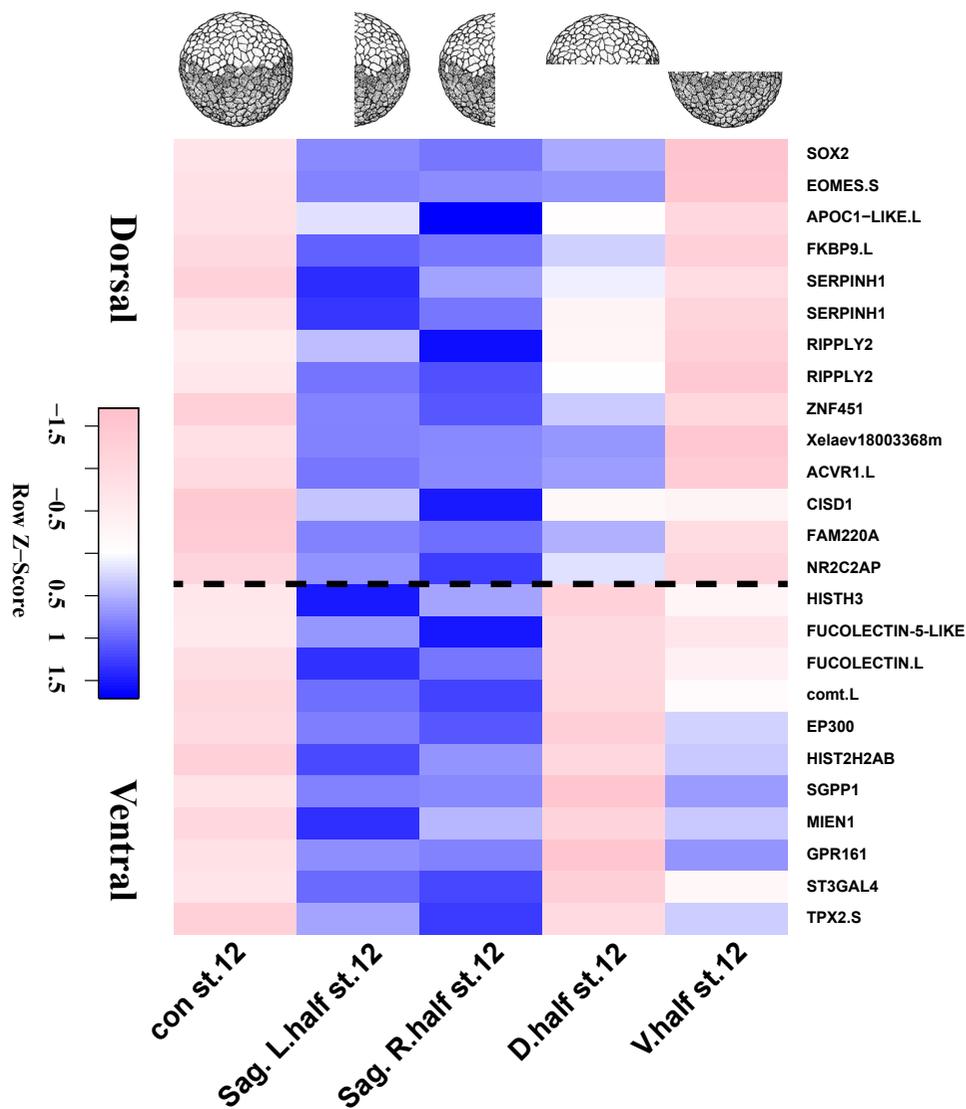*Listed according to average RPKM levels in control-embryos.

Fig. 2 Heatmap of genes up-regulated by sagittal bisection in regenerating stage 12 late gastrula *Xenopus laevis* embryos. *Heatmap showing the horizontal Row Z-scores of genes bisected at stage 8 and cultured until stage 12. Standard deviations were calculated from RPKMs of transcripts in left, right, and dorsal/ventral halves (see also Table 2). Highly expressed genes are shown in blue and lower ones in pink.*

sagittal half-embryos. They were all ventrally enriched transcripts, and found only in one embryo. Of the 11 genes, one lacked human homologues. ZNF268 is a zinc finger protein involved in human liver development (Sun *et al.*, 2004). ACAA2 is a liver enzyme in the fatty acid oxidation pathway (Sodhi *et al.*, 2014). Two other genes, CEBPD and UBP1, also regulate lipid metabolism (Lai *et al.*, 2017; Koutnikova *et al.*, 2009). ATP6V1A is a subunit of V-ATPase (Jing *et al.*, 2019). MCIDAS regulates cell cycle progression (Pefani *et al.*, 2011) and FOXI1 cell proliferation (Dou *et al.*, 1999). Calpain8 regulates cranial neural crest cell migration (Cousin *et al.*, 2010). HoxJ1 is transcription factor specifically required for the formation of motile cilia. Interestingly, HoxJ1 is down-regulated by Nanog (Siu *et al.*, 2013). Since HoxJ1 is down-regulated by sagittal section, this observation might be in line with the view that pluripotency may be sustained during self-regeneration.

The genome-wide analysis of transcripts decreased during twinning indicated that ventral genes involved in Chordin degradation, cell proliferation and lipid metabolism were down-regulated.

Discussion

The mechanism by which embryos self-organize to regenerate pattern has fascinated embryologists since the beginnings of experimental embryology (Driesch, 1891; Morgan, 1895; Spemann, 1938). In a recent study we reported that in bisected *Xenopus* embryos the Spemann organizer is formed 90⁰ away from its usual location (Moriyama and De Robertis, 2018). In that study, we were unable to identify any transcripts up-regulated specifically in sagittal bisections (when compared to D-V half-embryos in which wound healing genes are also activated) by analyzing groups of six pooled embryos. We now present the analysis of *individual* embryonic halves. Deep transcriptome analyses were possible due to the large size of the *Xenopus* embryo. The RNA-seq data from the 30 individual libraries bisected at mid-blastula and allowed to regenerate until gastrula stages 10.5 or 12 (available in supplemental tables S1 and S2 in a searchable Excel format, or as raw data at Gene Expression Omnibus database of the NIH, number GSE124563), provide a very rich resource to mine for genes regulated in the early vertebrate embryo. For example, all of the known Spemann organizer and ventral center genes (Reversade and De Robertis, 2005; De Robertis *et al.*, 2017), as well as others previously unknown, are strongly represented in the dorsal and ventral half-embryos bisected at stage 8 and allowed

embryo fragments at stage 10.5, mostly among ventrally-expressed genes. Many of them were novel genes. Among the identified genes, *DAPK2* is a positive regulator of programmed cell death (Yuasa, *et al.*, 2015). *DEGS2* plays a pivotal role in phytosphingolipid biosynthesis in skin and differentiation of human keratinocytes (Mizutani *et al.*, 2004). *TBX3*, is a T-box transcription factor crucial for mesendodermal differentiation in *Xenopus* (Weidgang, *et al.*, 2013). *TBX3* inhibits *BMP4* to regulate retina patterning (Motahari, *et al.*, 2016) and mutations of *TBX3* have been found to cause dominant autosomal ulnar-mammary syndrome (He *et al.*, 1999). *CRYGB* (crystallin gamma B) is a protein expressed in the early hindbrain and required for lens regeneration and development (Day and Beck, 2011). Finally, *PLSCR3* is a regulator of mitochondrial structure and respiration. (Liu *et al.*, 2003).

Table 4 shows genes decreased in both sides of individual

to regenerate until stage 12 (data available in Supplemental Table S2). Genes expressed on the dorsal fragment will include those responsible for the phenomenon known as scaling, by which a well-proportioned embryo of half the size is formed from the dorsal half embryo (Plouhinec *et al.*, 2013).

One main conclusion that emerged is from the analysis of up-regulated genes was that genes of the D-V morphogenetic pathway were not specifically overexpressed in regenerating twin embryos. This indicates that the displacement of the site of Spemann organizer formation may be achieved by non-transcriptional mechanisms during self-organization (Moriyama and De Robertis, 2018). In other words, our analysis did not reveal obvious master control genes up-regulated during regeneration at a transcriptional level. Diffusible molecules such as BMPs and Wnts may be sufficient to displace formation of the dorsal organizer, which self-regulates without large changes in the maximal expression levels of D-V genes (Fig. 1). However, the strong decrease in levels of *sizzled* expression during twinning suggests that Chordin protein levels might be increased indirectly, though the competitive inhibition of the Tolloid enzymes that degrade Chordin (Lee *et al.,* 2006; De Robertis *et al.*, 2017).

A drawback of our analysis is that we applied very stringent criteria to identify genes expressed differentially in sagittal versus D-V half embryos, to circumvent the activation of wound-healing genes. At early gastrula (4.5 hours after bisection) increased transcripts were mostly histones, presumably indicating a higher cell proliferation rate. Notably, at late gastrula (9 hours after bisection) several genes involved in stem cell pluripotency and germ layer determination (Sox2, Eomesodermin, Ripply 2, and ACVR1) were up-regulated specifically in sagittal twins (Fig. 2, Table2).

Importantly, the data presented here in Supplemental Tables S1 and S2 contains all transcriptome changes taking place at stages 10.5 and 12, and can be mined for many more additional interesting transcripts specifically up-regulated or down-regulated in experimentally twinned embryos simply by allowing for less stringent levels of differential expression. The data presented here represents a rich resource for the transcriptional changes that underlie twinning. The data for individual Dorsal and Ventral half embryos bisected at midblastula and allowed to regenerate until late gastrula offer the most exhaustive repertoire of D-V regulated genes available for a vertebrate embryo (supplemental Table S2).

Materials and Methods

Embryo manipulation and generation of sagittal half embryos
Xenopus laevis frogs were purchased from Nasco and all procedures were as approved by the animal research committee of the University of California, Los Angeles. Embryos were fertilized with sperm suspension (Sive *et al.*, 2000). Embryos with the best D-V polarity (Klein, 1987) were selected at the 2- and 4-cell stages, cultured in 0.1 × Marc's modified Ringer's and staged (Nieuwkoop and Faber, 1967). Stage 8 embryos were manually dechorionated with watchmaker forceps and bisected with an eyelash knife in 0.3 × Modified Barth's Solution (MBS, Gurdon, 1976) as described by Moriyama and De Robertis (2018). Sagittal bisections cut the dorsal crescent in half, while dorsal and ventral halves were sectioned perpendicular to that plane. Half embryos were rinsed to remove dead cells with a glass pipette and transferred within 3 min into depressions made in 2% agar-coated plates and allowed to heal for 60 min in 0.3 × MBS solution with the large wound facing upwards. Next, sibling half embryos were placed in fresh depressions in separate agar coated plates and cultured in 0.1 × MBS until they reached stage 10.5 or 12 (Nieuwkoop and Faber,

1967). The frequency of well-patterned twins was increased by choosing those embryos in which the large wound left by bisection was completed within 60 minutes, followed by a second screening for those sagittal half embryos in which the dorsal lip formed 90° away from the healing point. For stage 12 sagittally bisected embryos, a further selection was performed choosing half-embryos with asymmetric pigmentation on the left and right sides (see Moriyama and De Robertis, 2018).

RNA sequencing and data analysis
Total RNA was isolated from single whole or half embryos using the Absolutely RNA Miniprep Kit (Agilent). 500 ng of RNA were used to construct each library, using the Illumina TruSeq RNA Library Preparation Kit V2 following the manufacturer's protocol. Each library was sequenced in an Illumina Hi-SEq 2000 to generate 100-bp single-end reads at the Broad Stem Cell Research Center at the University of California, Los Angeles (UCLA). RNA sequencing data were processed as described previously (Anders and Huber, 2010; Love *et al.*, 2014; Ding *et al.*, 2017b). Reads were mapped against protein-coding regions in the JGI9.1 (XENLAv9.1) version of the *Xenopus laevis* genome (Session *et al.*, 2016) which is available at Xenbase. Human gene names (Tables S1 and S2) were assigned by blasting JGI9.1 peptide sequences to human reference protein sequences, as described previously (Ding *et al.*, 2017b). The RNA-seq data are publicly available at the N.I.H. Gene Expression Omnibus (GEO), accession no. GSE124563.

Heatmap
The heatmap in Fig. 2 was generated in R-Studio using RPKM of the indicated genes as input. Horizontal Row Z-scores were obtained from the RPKMs by calculating the mean, variance, and standard deviation of each transcript by using the gplots v3.0.1 package available from R-Studio. The rows/genes and columns/conditions were left unclustered.

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