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SUPPLEMENTARY MATERIAL

corresponding to:

Insulin-like growth factor-2 regulates early neural and cardiovascular system development in zebrafish embryos

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Supplementary Table 1
Classification of IGF-2 control embryos.

Uninjected control embryos			
	Normal	Mild	Dead
24 hpf	216/220	0/220	4/220
48 hpf	210/220	2/220	8/220
72 hpf	206/220	2/220	12/220
IGF-2a[1] control embryos			
	Normal	Mild	Dead
24 hpf	111/111	0/111	0/111
48 hpf	107/111	0/111	4/111
72 hpf	106/111	0/111	5/111
IGF-2b[2] control embryos			
	Normal	Mild	Dead
24 hpf	117/117	0/117	0/117
48 hpf	110/117	1/117	6/117
72 hpf	108/117	1/117	8/117
IGF-2(a+b)[3] control embryos			
	Normal	Mild	Dead
24 hpf	201/204	0/204	3/204
48 hpf	194/204	0/204	10/204
72 hpf	193/204	0/204	11/204

Embryos were injected with 4 ng IGF-2a[1] control morpholino, 4 ng IGF-2b[2] control morpholino or 2 ng IGF-2a and 2 ng IGF-2b[3] control morpholinos.

Supplementary Table 2
Classification of IGF-2 morphant embryos*.

IGF-2a[1] morphant embryos					
	Normal	Mild	Intermediate	Severe	Dead
24 hpf	85/119 (72%)	12/119 (10%)	12/119 (10%)	0/119 (0%)	10/119 (8%)
48 hpf	77/119 (65%)	15/119 (13%)	10/119 (8%)	0/119 (0%)	17/119 (14%)
72 hpf	66/119 (55%)	18/119 (15%)	14/119 (12%)	2/119 (2%)	19/119 (16%)
IGF-2b[2] morphant embryos					
	Normal	Mild	Intermediate	Severe	Dead
24 hpf	25/120 (21%)	15/120 (12%)	60/120 (50%)	11/120 (9%)	9/120 (8%)
48 hpf	19/120 (16%)	13/120 (11%)	69/120 (57.5%)	3/120 (2.5%)	16/120 (13%)
72 hpf	22/120 (18%)	17/120 (14%)	60/120 (50%)	1/120 (1%)	20/120 (17%)
IGF-2(a+b)[3] morphant embryos					
	Normal	Mild	Intermediate	Severe	Dead
24 hpf	23/257 (9%)	27/257 (11%)	119/257 (46%)	57/257 (22%)	31/257 (12%)
48 hpf	27/257 (11%)	3/257 (1%)	106/257 (41%)	43/257 (17%)	78/257 (30%)
72 hpf	23/257 (9%)	23/257 (9%)	90/257 (35%)	30/257 (12%)	91/257 (35%)

*The 24 hpf phenotypes are described in the results section of this paper. At 48 and 72 hpf mildly affected embryos exhibited at least one of the following: reduced head, shorter body, reduced eyes, reduced blood circulation or cardiac oedema. Intermediately affected embryos had a mild phenotype plus at least one of the following: disrupted brain structures, disrupted somites, increase in heart size, disrupted circulation, blood pooling, cardiac oedema or blood reflux. Severely affected embryos displayed a mild or intermediate phenotype plus at least one of the following: loss of brain structures, disrupted body plan or loss of circulating blood.
Embryos were injected with 4 ng IGF-2a[1], 4 ng IGF-2b[2] or 2 ng IGF-2a and 2 ng IGF-2b[3] morpholinos.

Supplementary Table 3

Description of angiogenesis defects in IGF-2 morphant embryos.

	Class I	Class II	Class III
26 hpf	ISV mildly reduced, slight expansion of ICM, basic outline of vasculature structure present in head but reduced in intensity compared to control injected embryos.	ISV sprouting disrupted, ICM expanded, vascular structures in head reduced or sprouting affected.	Primary vessels present but disorganised
48 hpf	ISV present, slight expansion of ICM, reduced intensity of GFP expression in all vascular structures (particularly in the head and eyes) compared to control embryos.	ISV sprouting disrupted, ICM expanded, less sprouting of vessels in head and eyes.	Primary vessels present but disorganised
72 hpf	ISV present, pattern of vasculature in head and eyes conserved but reduced sprouting of vessels, slight expansion of ICM, PAV reduced.	ISV disrupted, ICM expanded, sprouting of vessels in head and eyes reduced, PAV reduced or absent.	Primary vessels present but disorganised

Abbreviations: ISV, intersomitic vessels; ICM, intermediate cell mass; GFP, green fluorescent protein; PAV, parachordal vessel

Supplementary Table 4

Summary of IGF-2 morphant embryos displaying angiogenesis defects.

IGF-2a morphant phenotype[1]					
	Normal	Class I	Class II	Class III	Dead
26 hpf	24/60 (40%)	20/60 (33%)	13/60 (22%)	3/60 (5%)	
48 hpf	30/60 (50%)	13/60 (22%)	10/60 (17%)	4/60 (6%)	3/60 (5%)
72 hpf	36/60 (60%)	7/60 (11.6%)	7/60 (11.6%)	3/60 (5%)	7/60 (11.6%)
IGF-2b morphant phenotype[2]					
	Normal	Class I	Class II	Class III	Dead
26 hpf	10/52 (19%)	15/52 (29%)	20/52 (38.5%)	7/52 (13.5%)	
48 hpf	10/52 (19%)	11/52 (21%)	22/52 (42%)	3/52 (6%)	6/52 (12%)
72 hpf	12/52 (23%)	13/52 (25%)	15/52 (29%)	1/52 (2%)	11/52 (21%)
IGF-2(a+b) morphant phenotype[3]					
	Normal	Class I	Class II	Class III	Dead
26 hpf	11/82(13%)	17/82 (21%)	39/82 (48%)	15/82 (18%)	
48 hpf	14/82 (17%)	23/82 (28%)	23/82 (28%)	3/82 (4%)	19/82 (23%)
72 hpf	20/82 (24.4%)	21/82 (25.6%)	15/82 (18.3%)	1/82 (1.2%)	25/82 (30.5%)

Embryos were injected with 4 ng IGF-2a[1], 4 ng IGF-2b[2] or 2 ng IGF-2a and 2 ng IGF-2b[3] morpholinos.

Supplementary Table 5

Heart rates of control and IGF-2 morphant embryos at 72 hpf.

Embryo group	Mean heart rate	Standard deviation
Uninjected control	137	4
IGF-2a control	137	4
IGF-2b control	137	6
IGF-2(a+b) control	137	5
IGF-2a morphant	132	9
IGF-2b morphant	107	22
IGF-2(a+b) morphant	101	16

Supplementary Table 6

Oligonucleotide and morpholino sequences.

Oligonucleotides	**5' – 3'**
IGF-2a forward[1]	AAAATCGATATGGATGATTACCATGTATTC
IGF-2a reverse[1]	AATACGTATCATTTTCGGGATGTGCTG
IGF-2b forward[1]	AAAATCGATATGGAGGACCAACTAAAACAT
IGF-2b reverse[1]	AAATACGTATCACTTGTGGCTAACGTA
IGF-2aFSDM[2]	ATCCCATCGATATGGA**C**GATTA**T**CA**C**GTATT**T**TG**C**GCATCTTGCCGAA
IGF-2aRSDM	TCCATATCGATGGGATCCTGCAAAAAGAAC
IGF-2bFSDM[2]	ATCCCATCGATATGGA**A**GACCA**G**CT**G**AAACA**C**CA**C**TCTGTTTGCCATA
IGF-2bRSDM	TCCATATCGATGGGATCCTGCAAAAAGAAC
Morpholinos	
IGF-2a	CACAGAATACATGGTAATCATCCAT
IGF-2a control[3]	CAgAGAATAgATcGTAATgATCgAT
IGF-2b	AATGATGTTTTAGTTGGTCCTCCAT
IGF-2b control[3]	AATGATcTTTTAcTTGcTCgTCgAT

[1]Cla I and Snab I restriction sites are underlined.

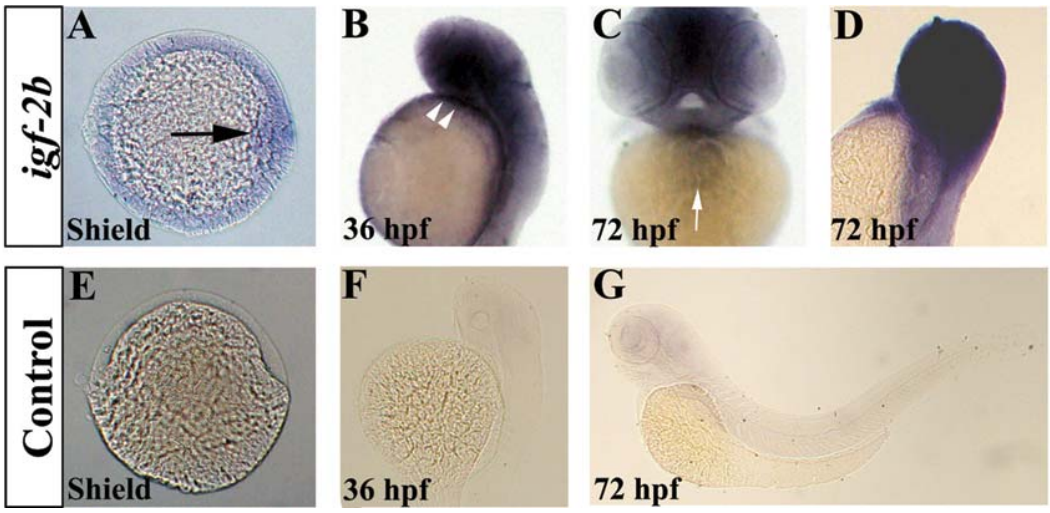
[2]Mutated sites are indicated in bold.

[3]Mismatches are indicated in lowercase.

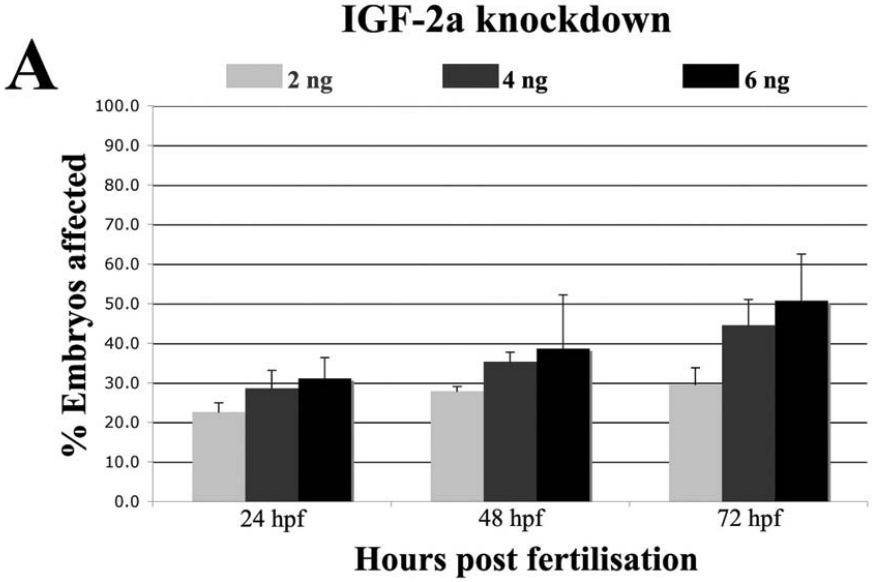
Supplementary Table 7

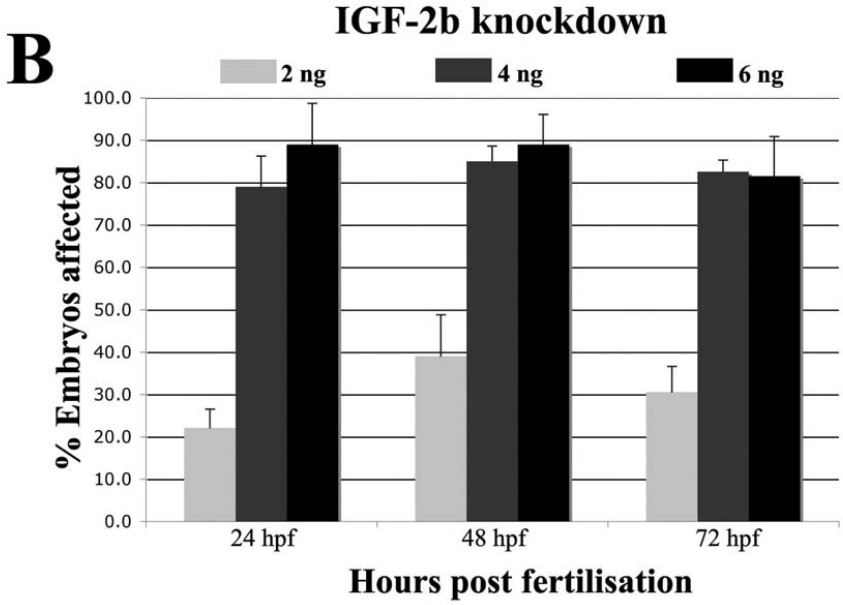
Numbers of control embryos displaying expression patterns as shown in figures.

	Uninjected control	**IGF-2a control**	**IGF-2b control**	**IGF-2(a+b) control**
pax6.2	29/29	29/29	30/30	30/30
rx3	32/32	29/29	28/28	31/31
fgf8	28/28	31/31	29/29	28/28
TUNEL	30/34	42/42	46/49	28/29
bozozok	27/27	52/52	58/58	62/62
chordin	31/31	34/34	22/22	28/28
goosecoid	25/25	28/28	28/28	49/49
bmp2b	31/31	35/35	24/24	30/30
bmp4 (shield)	48/48	56/56	56/56	55/55
gata2	47/47	31/31	32/32	34/34
O-dianisidine	33/33	30/30	30/30	27/27
scl (26 hpf)	28/28	48/48	60/60	59/59
gata1(26 hpf)	60/60	58/58	53/53	59/59
scl (22 somite)	69/69	69/69	54/54	54/55
gata1 (22 somite)	33/33	42/42	42/42	29/29
bmp4 (40 hpf)	23/23	29/29	26/26	25/25
notch1b	31/31	29/29	33/33	32/32
eln2	31/31	35/35	35/35	26/26
cmlc2	30/34	53/55	51/53	58/60

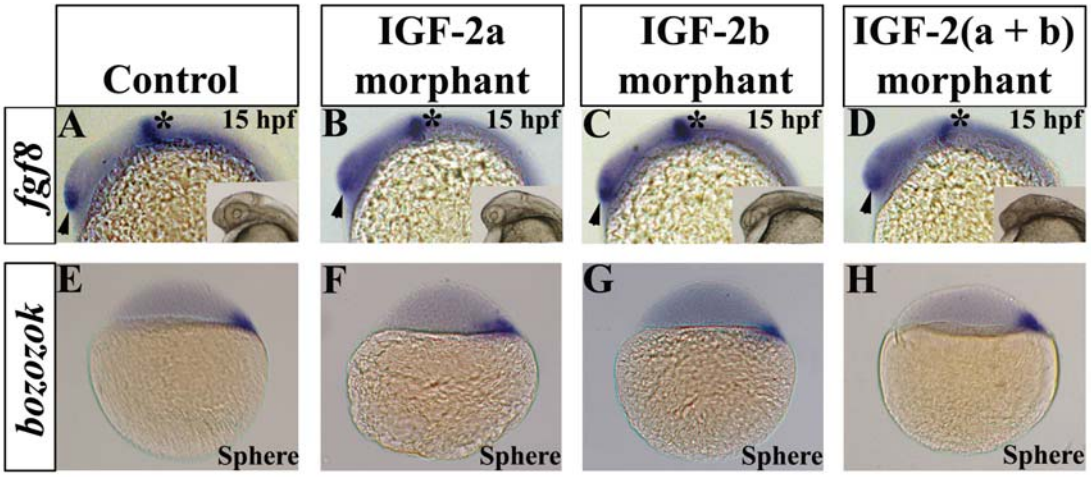


Supplementary Fig. 1. Expression of *igf-2b* during zebrafish embryogenesis. Whole mount *in situ* hybridisation was performed using *igf-2b*-specific antisense and sense probes. **(A)** *igf-2b* is expressed in the zebrafish embryonic shield (black arrow). **(B)** *igf-2b* is expressed in the developing anterior neural structures and the heart (white arrowheads). **(C, D)** By 72 hpf, *igf-2b* is expressed in the heart and in the anterior region of the embryo. **(E, F, G)** Embryos hybridised to the sense *igf-2b* probe. Frequency of embryos displaying this staining pattern; A, 51/55; B, 31/31; C, D, 26/26; E, 50/50; F, 28/28; G, 31/31. A, animal pole view of the embryo with dorsal to the right. B, D, E, F, G are shown in lateral view and C is shown in ventral view.

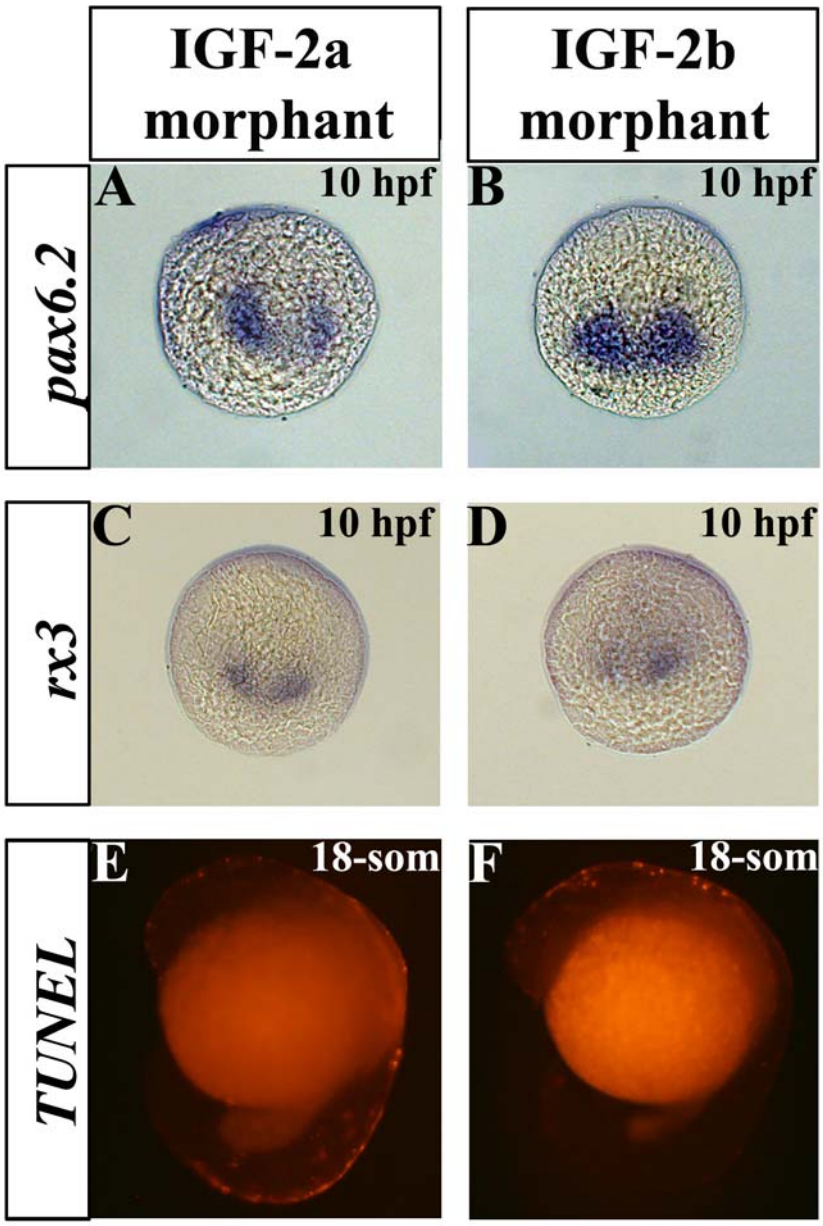




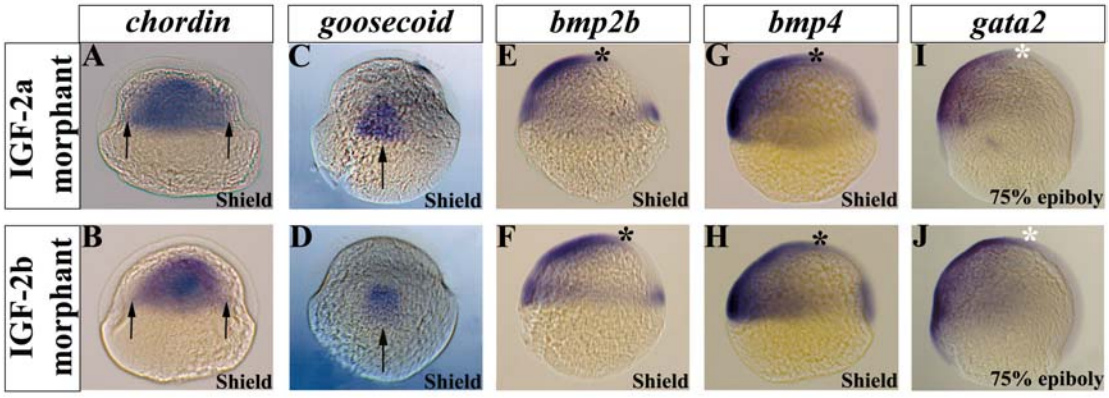
Supplementary Fig. 2. Effect of *igf-2a* or *igf-2b* knockdown on zebrafish development is dose dependent. (A, B) Data are presented as mean ± SD of three separate experiments (n ≥ 27 per experiment). Error bars indicate the standard deviation of the mean. Dose response experiments to identify an optimal dose of morpholino(s) were performed over three days. On each day approximately 60 embryos were injected with antisense or control morpholino(s). Injected embryos were visualised at 24, 48 and 72 hpf by light microscopy and classified as normal, mild, intermediate, severe or dead (n ≥ 100 embryos for each set of injections). The number of affected embryos is the sum of mild, intermediate, severe and dead embryos.



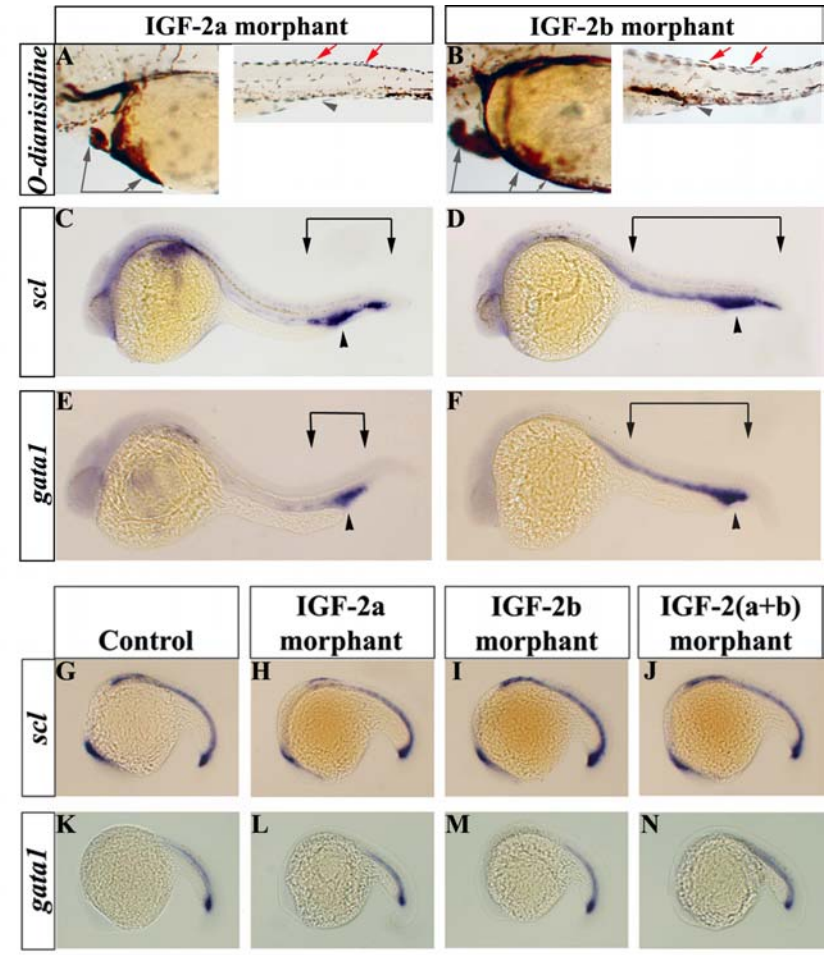
Supplementary Fig. 3. Expression of *fgf8* and *bozozok* in IGF-2 morphant embryos. (A-D) Knockdown of *igf-2a* and/or *igf-2b* does not disrupt patterning of the telencephalon (arrowhead) and the midbrain-hindbrain boundary (asterisk) during the segmentation stage in zebrafish development. (Inset) Anterior region of 24 hpf control and morphant embryos showing disrupted neural structures in morphants. **(E-H)** Expression of *bozozok* is unaffected in IGF-2 morphant embryos. Frequency of embryos displaying this staining pattern; B, 30/30; C, 28/28; D, 32/32; F, 60/60; G, 59/59; H, 42/42. All embryos are shown in a lateral view.



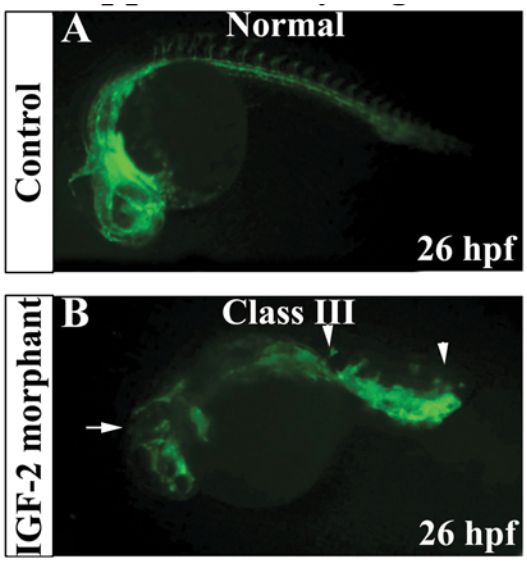
Supplementary Fig. 4. IGF-2a and IGF-2b are required for the development of anterior neural structures during zebrafish gastrulation and play anti-apoptotic roles during segmentation. (A, B) Knockdown of *igf-2a* or *igf-2b* results in a reduction of *pax6.2* expression in the developing eye and midline region. **(C, D)** Knockdown of *igf-2a* or *igf-2b* causes a reduction of *rx3* expression. **(E)** Knockdown of *igf-2a* results in an increase in apoptosis, particularly in the developing spinal cord. **(F)** Knockdown of *igf-2b* results in an increase in apoptosis, particularly in the anterior region of the embryo. Frequency of embryos displaying this staining pattern; A, 14/32; B, 8/20; C, 15/39; D, 14/33; E, 17/30; F, 12/33. A-D are views of the future anterior region and E, F are lateral views.



Supplementary Fig. 5. IGF-2a and IGF-2b regulates the expression of genes involved in dorsal-ventral patterning. (A, B) Expression of *chordin* is reduced in IGF-2a and IGF-2b morphant embryos. Arrows indicate the width of the *chordin* expression domain. **(C, D)** Expression of *goosecoid* is reduced in IGF-2a and IGF-2b morphant embryos (arrows). **(E-J)** Expression of *bmp2b, bmp4* and *gata2* is expanded towards the dorsal side of IGF-2a and IGF-2b morphant embryos (asterisks). Frequency of embryos displaying this staining pattern; A, 13/27; B, 17/35; C, 13/39; D, 17/31; E, 13/27; F, 16/32; G, 12/28; H, 15/36; I, 17/33; J, 21/39. All embryos are shown in a lateral view.



Supplementary Fig. 6. Defects in blood development are coincident with the onset of circulation in IGF-2 morphant embryos. (A, B) O-dianisidine stained IGF-2a and IGF-2b morphant embryos with reduced circulating blood at 72 hpf (heart and tail regions). Grey arrows indicate blood pooling under the heart, red arrows loss of blood circulation in the intersomitic vesicles and grey arrowheads the intermediate cell mass. **(C, E)** IGF-2a morphant embryos show an increase in *scl* and *gata1* expression at the intermediate cell mass. **(D, F)** Knockdown of *igf-2b* results in an increase in the region of *scl* and *gata1* and expression is expanded outside the intermediate cell mass. Arrowhead indicates position of intermediate cell mass and arrows indicate extent of expression along the embryo. **(G, K)** 22-somite stage embryos injected with control morpholino(s) showing normal *scl* and *gata1* expression. **(H-J, L-N)** Expression of *scl* and *gata1* is unaffected in IGF-2 morphant embryos before circulation commences. Frequency of embryos displaying this expression pattern: A, 20/64; B, 39/47; C, 12/43; D, 37/51; E, 19/75; F, 73/84; H, 32/32; I, 29/29; J, 35/35; L, 23/25; M, 29/31; N, 33/33. All embryos are shown in a lateral view.



Supplementary Fig. 7. Vasculogenesis is unaffected in IGF-2 morphant embryos. **(A)** Control *fli1:EGFP* transgenic embryo displaying normal vascular development at 26 hpf. **(B)** Class III IGF-2 morphant embryo displaying severe effects on angiogenesis, however the basic vasculature is laid down.

Supplementary Movie 1. Heart region of control injected embryo at 72 hpf. This shows normal cardiac contractility and circulation through the heart.

Supplementary Movie 2. A representative 72 hpf IGF-2a morphant heart. Note the increase in heart size, mild blood reflux, a decreased heart rate, pericardial oedema and reduced circulation.

Supplementary Movie 3. A representative 72 hpf IGF-2b morphant heart. Note the increase in heart size, blood reflux, a decreased heart rate, pericardial oedema and reduced circulation. The IGF-2(a+b) morphant heart has the same phenotype.