Appendix S1

Supplementary methods and results

Except where stated otherwise, embryos were raised at 28.5°C in 0.1× Hanks Balanced Salt Solution (HBSS) without Phenol Red (Sigma). At 24 hours post-fertilisation (hpf), PTU (1-phenyl-2-thiourea) was added to a final concentration of 0.003% w/v to prevent pigment formation.

Following overnight fixation of zebrafish embryos in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), whole-mount in situ hybridisation (WISH) was performed by standard methods, exactly as described in (Broadbent and Read, 1999). Proteinase K digestion prior to hybridisation was optimised for each new batch of enzyme. For 4 days post-fertilisation (dpf) embryos, digestion was continued until the tail-fin had just begun to disintegrate. For our current batch of enzyme, this required a 1 hour incubation in 20 μg/ml Proteinase K at 37°C. The probes used were full-length β*e1-globin* (GenBank accession AF082662), which was a gift from Andre Quinkertz (Quinkertz and Campos-Ortega, 1999), and full-length α*e1-globin* (IMAGE clone 6906170, GenBank accession BC071550). Clone identities were verified by sequencing.

For photography, embryos were mounted in 80% glycerol:20% PBS (with 0.1% Tween20). For sectioning, embryos were embedded in 1.5% agarose and soaked in 30% sucrose overnight. After freezing in liquid nitrogen, the blocks were equilibrated to –30°C and 15 μm sections were cut on a cryostat.

The peroxidase activity of haemoglobin was detected using 2,7-diaminofluorene (Fluka). This was performed as described in Weinstein et al. (Weinstein et al., 1996) except that the pre- and post-incubation fixation was in 4% paraformaldehyde in PBSA rather than in BT-fix. Staining times were extended to 2 hours at room temperature to allow staining of the more weakly expressing erythroblasts in addition to the strongly expressing primitive erythrocytes.

For tail amputations, embryos were anaesthetised with 0.016% w/v MS222 (Sigma) in 50:50 mixture of Liebovitz L-15 (Sigma) and 0.1× HBSS (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin) for 15 minutes prior to operation. The tail (all tissue posterior to the opening of the cloaca) was then amputated. Amputations were performed using a sapphire blade (World Precision Instruments) in order to minimise tissue damage to the embryo. Anaesthesia was maintained for 4 hours after operation, followed by transfer to fresh 0.1× HBSS with antibiotics for further development at 28.5°C. Most embryos rapidly re-established circulation to the trunk via development of arteriovenous shunts near the amputation site and mortality was minimal (greater than 95% survival to 7 dpf). Other than the (obvious) lack of tail, the development and behaviour of the embryos appeared normal, with the exception of delayed inflation of the swim bladder.

To obtain blood, anaesthetised embryos were exsanguinated then killed by over-anaesthesia. To prevent the rapid blood clotting that occurs in older embryos, this was performed in a 4:1 mixture of 0.9× SSC, 20 mM EDTA, 10 U/ml heparin in 1× Trypsin/EDTA (0.5 mg/ml Trypsin, 6 mM EDTA in PBS). The trunk was severed with a sapphire knife just anterior to the cloaca, and the blood cells were collected with a 2 μl pipette tip. For scoring of morphology, the cells were transferred to a haemocytometer slide. For each treatment, a minimum of 200 blood cells from each of five 7 dpf embryos were immediately scored as either definitive or primitive in morphology. For unoperated control embryos, an average of 94% of erythrocytes (range 92-97%) had definitive morphology, which is in agreement with Belair et al. (Belair et al., 2001). This proportion was

not reduced by removal of the tail at 1 dpf (average 96%, range 94-97%) or 3.5 dpf (average 96%, range 94-98%). Although we would not exclude a reduction in total numbers of erythrocytes following removal of the tail, it is clear that this tissue is not required for the switch from embryonic to definitive erythropoiesis. For photography, blood samples were placed on poly-L-lysine coated slides. After fixation with 4% PFA in PBSA (1 hour) and methanol (5 minutes) the slides were washed with water then mounted in 80% glycerol.

Additional references

Broadbent, J. and Read, E. M. (1999). Wholemount in situ hybridization of Xenopus and zebrafish embryos. *Methods Mol. Biol.* **127**, 57-67.

Quinkertz, A. and Campos-Ortega, J. A. (1999). A new beta-globin gene from the zebrafish, beta(E1), and its pattern of transcription during embryogenesis. *Dev. Genes Evol.* **209**, 126-131.