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*J Immunol* (2011) 186 (8): 4751–4761.

<https://doi.org/10.4049/jimmunol.1000343>

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# Suppressor of Cytokine Signaling 1 Regulates Embryonic Myelopoiesis Independently of Its Effects on T Cell Development

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Suppressor of cytokine signaling 1 (SOCS1) has been shown to play important roles in the immune system. It acts as a key negative regulator of signaling via receptors for IFNs and other cytokines controlling T cell development, as well as Toll receptor signaling in macrophages and other immune cells. To gain further insight into SOCS1, we have identified and characterized the zebrafish *socs1* gene, which exhibited sequence and functional conservation with its mammalian counterparts. Initially maternally derived, the *socs1* gene showed early zygotic expression in mesodermal structures, including the posterior intermediate cell mass, a site of primitive hematopoiesis. At later time points, expression was seen in a broad anterior domain, liver, notochord, and intersegmental vesicles. Morpholino-mediated knockdown of *socs1* resulted in perturbation of specific hematopoietic populations prior to the commencement of lymphopoiesis, ruling out T cell involvement. However, *socs1* knockdown also led to a reduction in the size of the developing thymus later in embryogenesis. Zebrafish SOCS1 was shown to be able to interact with both zebrafish Jak2a and Stat5.1 *in vitro* and *in vivo*. These studies demonstrate a conserved role for SOCS1 in T cell development and suggest a novel T cell-independent function in embryonic myelopoiesis mediated, at least in part, via its effects on receptors using the Jak2–Stat5 pathway. *The Journal of Immunology*, 2011, 186: 4751–4761.

The development and function of many cell lineages is controlled by cytokines and growth factors. These bind to specific cell-surface receptors that activate a range of intracellular signaling pathways, including the Jak-Stat module, to mediate their collective effects (1). An important facet of this process is the tight negative regulation of these signals, such as by suppressor of cytokine signaling (SOCS) proteins, which ensure signaling proceeds in a controlled manner (2). The eight members of the SOCS family, SOCS1–7 and Cis, all possess an N-terminal domain of variable length and weak conservation, a central Src

homology 2 (SH2) domain, and a highly conserved C-terminal domain known as the SOCS box (3). In addition, some SOCS members also possess a kinase inhibitory region (KIR) and extended SH2 subdomain (ESS) immediately preceding their SH2 domain (4, 5).

SOCS1 was discovered independently as a protein that, firstly, inhibited IL-6–mediated macrophage differentiation (6); secondly, bound to and inhibited the JH1 kinase domain of Jak2 (7); and thirdly, was recognized by a mAb directed against the SH2 domain of Stat3 (8). Other experiments revealed that transcription of *Socs1* was upregulated in response to several cytokines and growth factors both *in vitro* and *in vivo*, including IL-6 and IFN- $\gamma$  (6, 9–11). SOCS1 principally regulates cytokine signaling by directly binding to the activation loop of Jaks, with the KIR required for both high-affinity binding and pseudosubstrate inhibition of tyrosine kinase activity (4, 5, 7, 8), although its actions are also facilitated via docking to specific cytokine receptors, including IFN- $\gamma$  receptor (12).

SOCS1 is a key negative regulator of both innate and acquired immunity (13). *Socs1*-knockout mice develop a complex fatal neonatal disease characterized by fatty degeneration and necrosis of the liver and multiorgan failure as a result of inflammatory infiltration (10, 14, 15). These phenotypes appear largely due to hyperresponsiveness to IFN- $\gamma$  because they are ameliorated by genetic or Ab-mediated ablation of this cytokine (10, 16). However, *Socs1/IFN- $\gamma$*  double-knockout mice develop polycystic kidneys, chronic infections, and inflammatory lesions (16). These mice exhibit widespread disruption of T cell development, including decreased numbers of T cells and reduced CD4/CD8 ratio (17), perturbed Th2 responses (18), and defective Th17 development (19) caused by additional hypersensitivity to cytokines acting via the  $\gamma_c$  receptor, including IL-2, IL-4, IL-7, IL-15 (20), as well as IL-12 (21). SOCS1 deficiency also results in excessive macrophage and dendritic cell activation (22, 23), likely due to the

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Received for publication February 1, 2010. Accepted for publication February 1, 2011.

This work was supported by an Australian postgraduate award (to L.A.O. and M.K.T.), Deakin University postgraduate research awards (to R.S.L. and C.L.), a National Health and Medical Research Council R.D. Wright Fellowship (to S.E.N.), and the Malaysian Ministry of Higher Education and the University of Malaya, Kuala Lumpur, Malaysia (to S.M.N.). This work used the resources of the Australian Research Council Linkage-Equipment Infrastructure Fund-supported *FishWorks: Collaborative Infrastructure for Zebrafish Research* and was funded by an Australian Research Council Discovery grant.

The zebrafish *socs1* promoter presented in this article has been submitted to GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) under accession number BN000985.

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Abbreviations used in this article: ESS, extended Src homology 2 subdomain; EST, expressed sequence tag; hpf, hours postfertilization; KIR, kinase inhibitory region; LPM, lateral plate mesoderm; MO, morpholino; pICM, posterior intermediate cell mass; SCR, scrambled; SH2, Src homology 2; SOCS1, suppressor of cytokine signaling 1; UTR, untranslated region; WISH, whole-mount *in situ* hybridization; wt, wild-type.

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combined effects of unrestrained signaling via IFN $\gamma$ R1 (24) and TLRs (25, 26).

Zebrafish has emerged as a valuable model for the study of vertebrate development, with the expression and function of the genes involved in the development of innate and acquired immunity particularly analogous between zebrafish and mammals (27, 28). This extends to components of the cytokine receptor–Jak–Stat–SOCS pathway (29–35), analysis of which has often revealed new insights into their function (36, 37). Therefore, we sought to investigate *socs1* function in zebrafish to augment the previous work performed in mammalian systems. In this study, we have identified and characterized zebrafish *socs1*, which showed high conservation to mammalian *Socs1*. Analysis of its embryonic expression pattern revealed broad domains of expression, including areas of hematopoietic and immune system development. Consistent with this, specific knockdown of the *socs1* gene revealed a novel, T cell-independent function in embryonic myelopoiesis that is likely mediated via its action on the Jak2–Stat5 pathway, as well as confirming a prominent role in T cell development.

## Materials and Methods

### Identification and characterization of *socs1* homologs from fish

Zebrafish (*Danio rerio*) expressed sequence tags (ESTs) encoding a protein with high homology to murine SOCS1 were identified by BLASTX searching. A putative full-length cDNA IMAGE clone (ID 3733230) in pSPORT1 was obtained from Invitrogen (Mount Waverley, VIC, Australia) and sequenced in full. This sequence was used to identify ESTs encoding a putative *socs1* ortholog from pufferfish (*Takifugu rubripes*). Both sequences were deposited at GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) with the following accession numbers: AJ972922, zebrafish *socs1*, and BN000784, pufferfish *socs1*. Analysis of zebrafish *socs1* expression was achieved by RT-PCR using intron-spanning primers for *socs1* (5'-TGGAA-GCGGCGACGAGAGTT-3' and 5'-CGGCTTGAAATGTGTCTGG-3') and  $\beta$ -actin (38) on RNA extracted from zebrafish embryos or microdissected adult tissue samples using TRIzol (Invitrogen).

### Bioinformatic analysis

Phylogenetic analyses and multiple alignments were performed as previously described (31). GenBank accession numbers of proteins included in the analysis were: NP\_003736, *Homo sapiens* SOCS1; NP\_003736, *Mus musculus* SOCS1; NP\_665886, *Rattus norvegicus* SOCS1; NP\_003946, *H. sapiens* SOCS3; NP\_031733, *M. musculus* SOCS3; NP\_446017, *R. norvegicus* SOCS3; AAH54214, *Xenopus laevis* SOCS1; NP\_062628, *M. musculus* SOCS5; NP\_724096, *Drosophila melanogaster* SOCS36E; NP\_523659, *D. melanogaster* SOCS44A; NP\_523390, *D. melanogaster* SOCS16D; CAJ00199, *Takifugu rubripes* SOCS1; CAI99889, *D. rerio* SOCS1; NP\_032439, *M. musculus* Jak2; NP\_571168, *D. rerio* Jak2a; and XP\_690070, *D. rerio* Jak2b.

### Analysis of zebrafish genomic sequences

Genomic sequences corresponding to the zebrafish *socs1* gene were identified by BLASTN searching of assembled zebrafish genomic sequences at the Sanger Centre ([http://www.ensembl.org/Danio\\_rerio](http://www.ensembl.org/Danio_rerio)) and the zebrafish WGS trace repository at GenBank (<http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html>). These were collectively assembled into contigs using Sequencher (Gene Codes) and the positions of intron/exon boundaries determined by alignment with the corresponding zebrafish *socs1* cDNA sequence, applying the GT-AG rule (39). The zebrafish *socs1* promoter was deposited at GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) under accession number BN000985.

### Mapping of the zebrafish *socs1* gene

The chromosomal location of the zebrafish *socs1* gene was determined using the Radiation Hybrid Mapping service at the Zon laboratory (<http://zfrhmaps.tch.harvard.edu/Zon/RHmapper>) and a region derived from the 3' untranslated region (UTR) of the gene. The positions of surrounding genes were determined by analysis of the Washington University Zebrafish Genomic Resources integrated map ([http://www.genetics.wustl.edu/fish\\_lab/frank/cgi-bin/fish](http://www.genetics.wustl.edu/fish_lab/frank/cgi-bin/fish)) and the Zebrafish Information Network integrated ZMAP

(<http://zfin.org>). The genomic arrangement of the mouse *Socs1* gene locus on chromosome 16p12-p13.1 (40), along with relevant genes on chromosome 17, was determined using the National Center for Biotechnology Information map viewer Web site (*M. musculus* Build 34.1) (<http://www.ncbi.nlm.nih.gov/mapview/>).

### Cloning

A construct encoding zebrafish SOCS1 with an N-terminal FLAG (DYKDDDDK) epitope tag, and lacking UTR MO targeting sequences, was generated by PCR from the original *socs1* IMAGE clone to generate a fragment with in-frame AscI and MluI restriction sites at the N and C termini that was subcloned into the mammalian expression vector pEF-FLAG-1, a derivative of pEF-BOS (41). The FLAG-*socs1* gene was subsequently amplified with primers incorporating HindIII and BamHI restriction sites at the 5' and 3' region of the gene, respectively, and cloned into the multiple cloning site of pBK-CMV (Stratagene, La Jolla, CA).

### Zebrafish

Wild-type (wt) stocks of zebrafish were sourced from St. Kilda Aquarium (St. Kilda, VIC, Australia) and MAS imports (Coburg, VIC, Australia) and maintained using standard practices, as previously described (42). All experiments performed were approved by the Deakin University Animal Welfare Committee.

### Whole-mount in situ hybridization and histological analysis

Whole-mount in situ hybridization (WISH) analyses were performed as previously described (42). A digoxigenin-labeled antisense riboprobe targeting the 3' half of the *socs1* gene was produced by linearizing the IMAGE clone using EcoRI digestion and in vitro transcription with SP6 polymerase. A sense probe to the same region of the gene was produced by deleting the 5' half of the gene using the internal EcoRI restriction enzyme. A digoxigenin-labeled sense riboprobe was prepared by linearizing this construct using BamHI and in vitro transcription with T7 polymerase. Other probes used were *lmo2* (43), *gata1* (44), *spil* (45), *ikaros* (46), *rag1* (47), *lyz* (48), *mmp9* (49), *fms* (50), *l-plastin* (51), and *mpx* (52). Some hybridized embryos were sectioned and counterstained with Fast Red, as described previously (49). Blood cells were visualized histologically by Wright-Giemsa staining of cytospin preparations (53) or O-dianisidine hemoglobin staining of whole embryos (52).

### Morpholino knockdown and rescue

Two nonoverlapping antisense morpholinos (MOs) were designed to target the zebrafish *socs1* gene: ATG MO targeting the start codon (5'-CC-*ACTGA*ACTGTGCGCCACCATCCT-3', 25 bp, 16% guanine, start codon italicized) and UTR MO targeting the 5' UTR (5'-CCAACCGCGTG-GTCTTCCATACTG-3', 25 bp, 24% guanine). A control scrambled MO (SCR MO; 5'-CCTCTTACCTCAGTTACAATTATA-3', 25 bp, 4% guanine) was also injected. These were diluted to 1.0 mM in 1 $\times$  Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM CaCl<sub>2</sub>, and 5.0 mM HEPES [pH 9]) and injected into one to eight cell embryos, which were allowed to develop. For phenotypic rescue experiments, the *socs1* UTR MO was coinjected with 50 pg synthetic mRNA encoding FLAG-*socs1* transcribed in vitro using the T7 mMessage mMachine Kit (Ambion, Austin, TX). In other experiments, a previously described *stat5.1* Splice MO (54) was coinjected, or embryos were treated with the Jak2 inhibitor, AG490 at 50  $\mu$ M (33), as described.

### EMSA

Human 293T cells were transiently transfected using Fugene with constructs expressing FLAG-tagged zebrafish SOCS1, Stat5.1 (53) and hyperactive E629K mutant Jak2a (33), and nuclear extracts prepared and analyzed by EMSA as described previously (55). Double-stranded probes corresponded to the promoter of  $\beta$ -casein (5'-AGATTTCTAGGAAT-CAAATC-3') or zebrafish *socs1* (5'-ATTTCTGGAAACAG-3').

### Luciferase assay

Human 293T cells were transiently transfected with constructs expressing FLAG-tagged murine and zebrafish SOCS1 proteins, murine Gp130, and a Stat3-responsive firefly luciferase reporter gene (APRE-luc), as described previously (56). To control for transfection efficiency, cells were transfected with a vector expressing Renilla luciferase downstream of a herpes simplex virus thymidine kinase (pRL-TK; Promega, Madison, WI). Cells were incubated overnight with 10 ng/ml human LIF (AMRAD, Melbourne, VIC, Australia) and a Dual-Luciferase assay performed (Promega) following the manufacturer's recommendations. Samples were analyzed using

a Microtiter plate luminometer (LUMIstar Galaxy; BMG Labtechnologies, Offenburg, Germany). LIF-induced firefly luciferase activity was normalized to the level of Renilla luciferase activity.

#### *In vitro* transcription and translation of zebrafish *Soxs1*

Coupled *in vitro* transcription-translation was carried out using the TNT-T7 Quick Coupled Transcription/Translation Systems (Promega). One microgram *soxs1* plasmid template DNA along with 100 ng either *soxs1* UTR MO or SCR MO was used in a 50  $\mu$ l reaction mixture, along with a control in which no DNA was added, and incubated for 90 min at 30°C.

#### Western blot analysis

*In vitro* translation products were analyzed via SDS-PAGE, transferred to polyvinylidene difluoride, and detected using the Transcend Non-Radioactive Translation Detection System (Promega). Western blot analysis on transfected 293T cells was performed as described previously (57). Lysates were probed with a polyclonal rat anti-FLAG Ab (a kind gift of Drs. D. Huang and L. O'Reilly, The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia), which was directly conjugated with HRP (Boehringer Mannheim Biochemica, Mannheim, Germany).

## Results

### *Identification and characterization of the zebrafish soxs1 gene*

Database searching with the murine SOCS1 sequence identified several homologous zebrafish EST clones. From these, a putative full-length zebrafish cDNA clone was identified and sequenced. This information was then used to extract a putative SOCS1 homolog from pufferfish. The conceptual translations of both fish genes showed high sequence identity with mammalian SOCS1 proteins, including the presence of a KIR, ESS, SH2 domain, and SOCS box in each case (Fig. 1A). Phylogenetic analysis of full-length sequences confirmed that the fish proteins formed a clade with known mammalian SOCS1 proteins, with high bootstrapping values. This clade was distinct from that of the closely related SOCS3 proteins, as well as other SOCS proteins, including those from fruit fly (Fig. 1B). In the absence of appropriate zebrafish reagents, we tested the ability of zebrafish SOCS1 to inhibit murine Gp130 signaling, using an assay in which LIF signaling through the Gp130 receptor can be quantified using a Stat3-luciferase reporter (56). In the absence of SOCS proteins, the luciferase activity from the Stat3-luciferase reporter was markedly increased in response to LIF stimulation compared with unstimulated cells (Fig. 1C, upper panel). The addition of murine SOCS1 inhibited LIF-induced luciferase activity, as previously reported (56). When expressed at similar levels (Fig. 1C, lower panel), zebrafish SOCS1 also suppressed this activity to at least the same extent as murine SOCS1 (Fig. 1C, upper panel), suggesting functional conservation. The zebrafish gene also showed conservation with mammalian *Soxs1* at the genomic level. This was reflected in the intron-exon structure, with a single intron in the 5' UTR of both zebrafish and mouse genes (Fig. 1D), the presence of an upstream open reading frame that is terminated one nucleotide prior to the initiation codon (Fig. 1E), and syntenic relationships, with the Riken LOC74374 gene located immediately downstream in a tail-to-tail orientation in both zebrafish and mouse (Fig. 1F). RT-PCR detected *soxs1* transcripts throughout embryogenesis (data not shown). Exhaustive analysis of genomic and EST sequences revealed no evidence of gene duplication. This collectively suggests that the zebrafish gene identified was an authentic SOCS1 ortholog.

### *Zebrafish soxs1 is widely expressed during embryogenesis*

Analysis of *soxs1* expression during zebrafish embryonic development by RT-PCR showed initial maternal derivation, followed

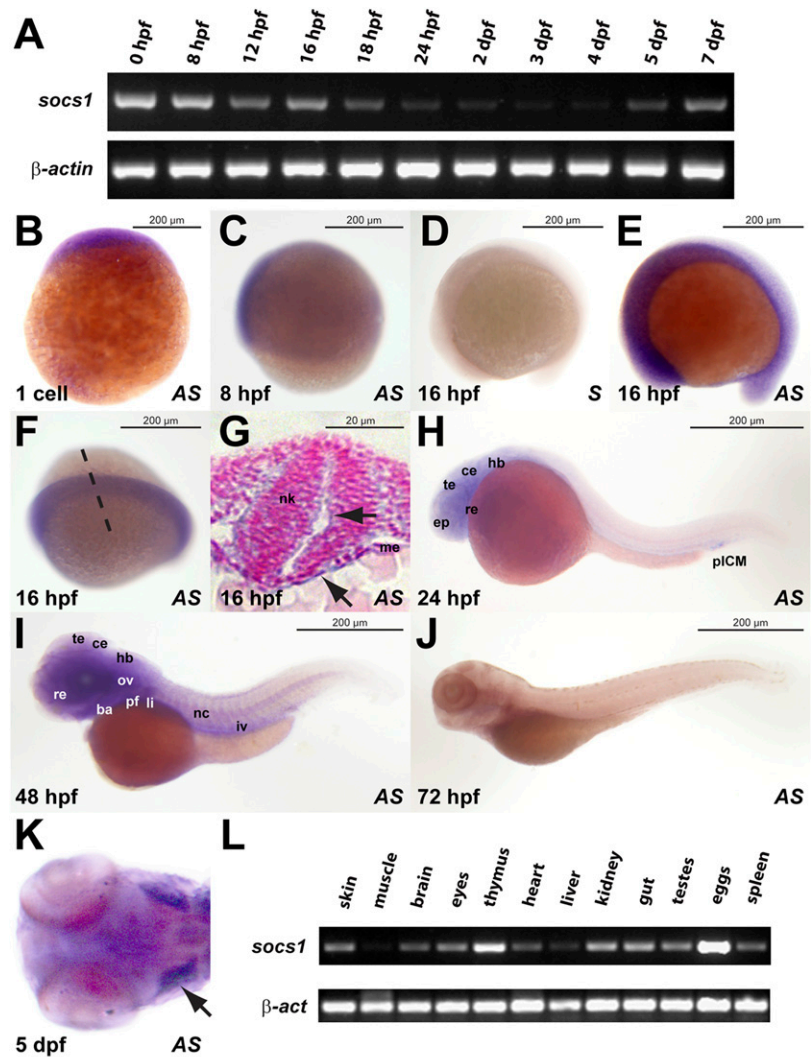
by ongoing zygotic expression throughout embryogenesis (Fig. 2A). WISH of staged embryos using an antisense probe targeting the 3' half of the zebrafish *soxs1* gene confirmed that transcripts were evident at the one-cell stage (Fig. 2B) and continued to be uniformly distributed until the gastrula period (8 hours post-fertilization [hpf]), when the expression domain became predominantly contracted to the mesoderm (Fig. 2C), presumably due to differential zygotic expression of the gene. At 16 hpf, *soxs1* expression continued to be concentrated in the mesoderm and epidermal cells surrounding the neural keel as confirmed by sectioning the embryo (Fig. 2E, 2G). In the pharyngula period (from 24 hpf), weak expression was observed in the lateral plate mesoderm (LPM)-derived posterior intermediate cell mass (pICM), the major site of primitive hematopoiesis at this time (58). Strong expression of *soxs1* was also seen in the head, especially in the telencephalon, cerebellum, hindbrain, retina, and epiphysis (Fig. 2H). During the hatching period (around 48 hpf), the strong anterior expression of *soxs1* continued, with additional domains of expression in the liver, notochord, and intersegmental blood vessels (Fig. 2I), but none at hematopoietic sites, such as the caudal hematopoietic tissue. At 72 hpf, embryonic expression of *soxs1* was no longer strongly evident (Fig. 2J), but specific staining was seen in later embryos in a region that includes the developing thymus (Fig. 2K). A corresponding sense probe control gave no staining at any time point (Fig. 2D and data not shown). Analysis of adult tissue samples by RT-PCR showed broad expression, including strong expression in the kidney and thymus (Fig. 2L).

### *Targeted knockdown of zebrafish soxs1 perturbs the pICM*

To directly assess the *in vivo* function of zebrafish SOCS1, the *soxs1* gene was targeted with antisense MO oligonucleotides (59). The *soxs1* gene does not contain an intron in the coding region of the gene, and so splice-site blocking MO (60) could not be used. Therefore, two nonoverlapping translation-blocking MO were used: one directed to the 5' UTR of the *soxs1* gene (UTR MO) and one directed to the sequence around the start codon (ATG MO) (Fig. 3A). To confirm the efficacy and specificity of this approach, SOCS1 was expressed using a coupled *in vitro* transcription-translation system. A single major band corresponding to SOCS1 (~25 kDa) was produced in presence of an SCR MO, which was substantially reduced in the presence of the UTR MO (Fig. 3B) or ATG MO (data not shown).

The *soxs1* UTR MO and ATG MO were delivered via microinjection into one to eight cell embryos, which were subsequently monitored throughout development in comparison with embryos injected with SCR MO or diluent only. Initial injections lead to a very high incidence of embryonic death, accompanied by severe dorsalization. Therefore, the *soxs1* MO concentrations were titrated to a level at which no developmental phenotypes or loss of viability were observed at early time points following injection. At these concentrations, both MO produced a clear enlargement of the area around the pICM from 20 hpf in injected embryos (Fig. 3D and 3E compared with 3C), which was not observed with SCR MO or diluent only (Table I). A limited number of embryos also exhibited subtle anterior defects that were not investigated further (data not shown). To confirm the specificity of the pICM phenotype, the UTR MO was coinjected with a synthetic capped mRNA encoding FLAG-tagged SOCS1 and lacking the MO target sequence. This largely rescued the phenotypes caused by the UTR MO (Fig. 3F), with the number of embryos exhibiting pICM expansion reduced to 3–5% ( $n = 295$ , three injections). The expansion was transient and no longer apparent in either this area or the caudal hematopoietic tissue (61), which develops adjacent from 36 hpf.





**FIGURE 2.** Zebrafish *socs1* is widely expressed. *A*, RT-PCR analysis of *socs1* expression during embryogenesis. Total RNA extracted from zebrafish embryos at the indicated time points and subjected to RT-PCR with primers specific for *socs1* (upper panel) or  $\beta$ -actin (lower panel). *B–K*, WISH of zebrafish embryos. Antisense (AS; *B*, *C*, *E–K*) and sense (S; *D*) *socs1* probes were used on embryos at the following ages: one cell (animal pole toward top) (*B*); 8 hpf (lateral view) (*C*); 16 hpf (lateral view) (*D*, *E*); 16 hpf (oblique dorsal view) (*F*); 16 hpf (transverse cross section along the plane indicated by dotted line in *F* and stained with nuclear red) (*G*); 24 hpf (lateral view) (*H*); 48 hpf (lateral view) (*I*); 72 hpf (lateral view) (*J*); and 5 dpf (dorsal view) (*K*). Where applicable, anterior is oriented toward the left. *L*, RT-PCR analysis of *socs1* in the adult. RNA extracted from the adult tissues indicated were analyzed as described in *A*. ba, branchial arches; ce, cerebellum; ep, epiphysis; hb, hindbrain; iv, intersegmental vesicles; li, liver primordium; me, mesoderm; nc, notochord; nk, neural keel; ov, otic vesicle; pf, pectoral fin; re, retina; te, tectum; with areas of staining in 16 hpf and 5 dpf embryos indicated with arrows.

#### *socs1*-knockdown disrupts embryonic hematopoiesis

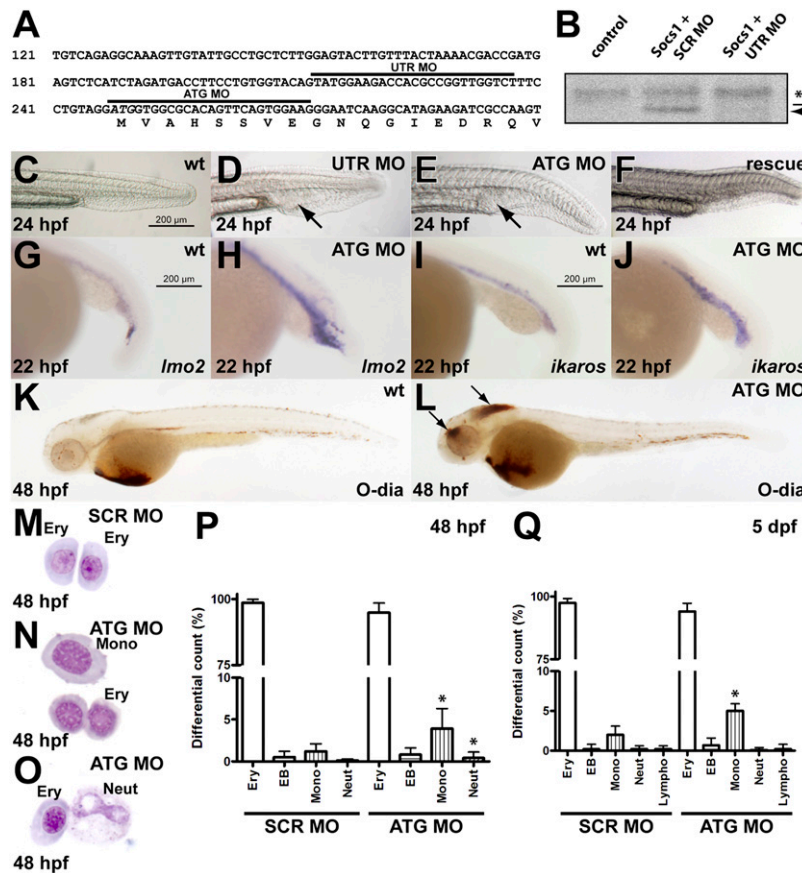
The pICM is one of the key sites of embryonic primitive hematopoiesis in zebrafish (62). Therefore, to further explore potential hematopoietic defects, embryos were probed with markers specific for early hematopoietic progenitors at 22 hpf, including *lmo2* (43) (Fig. 3*G*, 3*H*) and *ikaros* (46) (Fig. 3*I*, 3*J*). Morphant embryos showed an expansion in this cell compartment concomitant with pICM enlargement. By 48 hpf, embryos showed some evidence of hemorrhages by light microscopy and were stained with *O*-dianisidine to visualize RBCs (Fig. 3*K*, 3*L*). This confirmed the presence of hemorrhages in *socs1* morphants, particularly around the eyes and in the brain, with slightly reduced overall staining. The integrity of the vasculature was examined using *flk-1* as a marker (63). However, no specific vascular defects were observed in morphant embryos compared with controls (data not shown). Analysis of blood cytospin preparations at 48 hpf (Fig. 3*M*, 3*N*) revealed a statistically significant increase in the frequency of monocyte/macrophages and neutrophils (Fig. 3*P*), with the WBC/RBC ratio increased from 1.3 to 4.3%. The erythrocyte

population was also perturbed in *socs1* morphants, manifested by an increased nucleus/cytoplasm ratio in 5–10% of cells (e.g., Fig. 3*N*). The increased frequency of monocyte/macrophages was maintained at 5 dpf (Fig. 3*Q*).

Distinct hematopoietic cell populations were further characterized using WISH analysis with lineage-specific probes (48). Consistent with the increased numbers of hematopoietic progenitors, there was also modest expansion in the cell population derived from the posterior LPM expressing the early erythroid marker *gatal* (44) (Fig. 4*A*, 4*B*) or that derived from the anterior LPM expressing the early myeloid marker *spi1* (45) (Fig. 4*C*, 4*D*) in around 25–30% of embryos. At 48 hpf, a similar proportion of embryos showed a strong increase in the numbers of cells expressing the heterophil cell marker *mmp9* (49) (Fig. 4*G*, 4*H*), with effects on cells expressing the leukocyte markers *l-plastin* (51) (Fig. 4*E*, 4*F*) and *lyz* (48) (Fig. 4*I*, 4*J*) being more modest. By 72 hpf, cells expressing *fms* (50) (Fig. 4*Q*, 4*R*) and *mmp9* (Fig. 4*S*, 4*T*, 4*V*), remain expanded, with the *mpx* (52) (Fig. 4*K*, 4*L*) and *lyz* (Fig. 4*M–P*, 4*U*) populations unaffected. Lymphopoiesis only

nucleotides. *E*, Analysis of upstream open reading frames. Nucleotides are shown with numbering at the left and encoded amino acids indicated below. Regular text indicates upstream open reading frames, and bolded text indicates the *Socs1* open reading frames in mouse and zebrafish. *F*, Syntenic analysis of zebrafish and mouse *Socs1* gene loci. The relative orientation and approximate sizes of *Socs1* and surrounding genes located on mouse chromosomes 16 and 17, compared with zebrafish chromosome 3, are indicated.





**FIGURE 3.** Targeted knockdown of zebrafish *socs1* perturbs the site of embryonic hematopoiesis. *A*, Sequence targeted by *socs1* MO. Nucleotides 121–300 of the zebrafish *socs1* gene are shown with numbering at the left and encoded amino acids indicated below. The relative positions of the MO are indicated with a line above the sequence: UTR MO, targeting the 5' UTR; and ATG MO, targeting the start codon (italicized). *B*, Suppression of *socs1* translation by UTR MO. Coupled in vitro transcription-translation in the presence of no DNA (control), zebrafish *socs1* cDNA, and scrambled MO (SOCS1 + SCR MO) or zebrafish *socs1* cDNA and UTR MO (SOCS1 + UTR MO). The arrow indicates specific band in the SOCS1 + SCR MO lane; the asterisk indicates nonspecific band in all lanes, and the line denotes relative position of 25 kDa size marker. *C–F*, Visual analysis of *socs1* morphants. Light microscopic images of uninjected wt embryos (*C*) or embryos injected with UTR MO (*D*), ATG MO (*E*), or ATG MO plus in vitro transcribed, MO-resistant *socs1* mRNA (*F*) at 24 hpf. All embryos are lateral view with anterior to the left. Expansion of the posterior ICM at 24 hpf is indicated with arrows (*D*, *E*). *G–J*, WISH with pICM specific markers. Wild-type (diluent or SCR MO-injected) embryos (*G*, *I*) or embryos injected with ATG MO (*H*, *J*) were analyzed by WISH at 22 hpf with *lmo2* (*G*, *H*) and *ikaros* (*I*, *J*). All embryos are lateral view, anterior toward the left. The following markers were used, both at 22 hpf. *K* and *L*, Visualization of RBCs in *socs1* morphants. Wild-type (diluent or SCR MO-injected) embryos (*K*) or embryos injected with ATG MO (*L*) were stained with *O*-dianisidine (*O*-dia) at 48 hpf to visualize RBCs, with areas of hemorrhage indicated by arrows. *M–Q*, Blood cell analysis. Cytospin preparations of blood samples taken from pooled zebrafish embryos at the times indicated and stained with Wright-Giemsa (*M*, *O*) and then quantified (*P*, *Q*). EB, erythroblast; Ery, erythrocyte; Lympho, lymphocyte; Mono, monocyte/macrophage; Neut, neutrophil.

commences at 3 dpf in zebrafish (64) and was investigated using *ikaros* and *rag1*, which mark lymphoid precursors and mature lymphoid cells, respectively, in the developing thymus (47). The

Table I. Quantitation of key phenotypes observed in *socs1* morphants

	wt (%)	<i>socs1</i> MO (%) <sup>a</sup>	
Expanded pICM	24 hpf	0	36.8
Hemorrhage	48 hpf	0	29.1
Increased <i>spi1</i> <sup>+</sup> cells	22 hpf	2.8	25.8
Increased <i>mmp9</i> <sup>+</sup> cells	48 hpf	4.3	39.7
	72 hpf	5.8	42.9
Reduced <i>ikaros</i> staining	72 hpf	3.8	52.0
Reduced <i>rag1</i> staining	96 hpf	2.7	48.1

Proportion of embryos exhibiting each phenotype was defined as >1 SD from the mean observed in the wt embryos with respect to area (pICM, *ikaros*, and *rag1* staining) or number of expressing cells (*spi1*, *mmp9*), or simply the presence of the phenotype (hemorrhage).

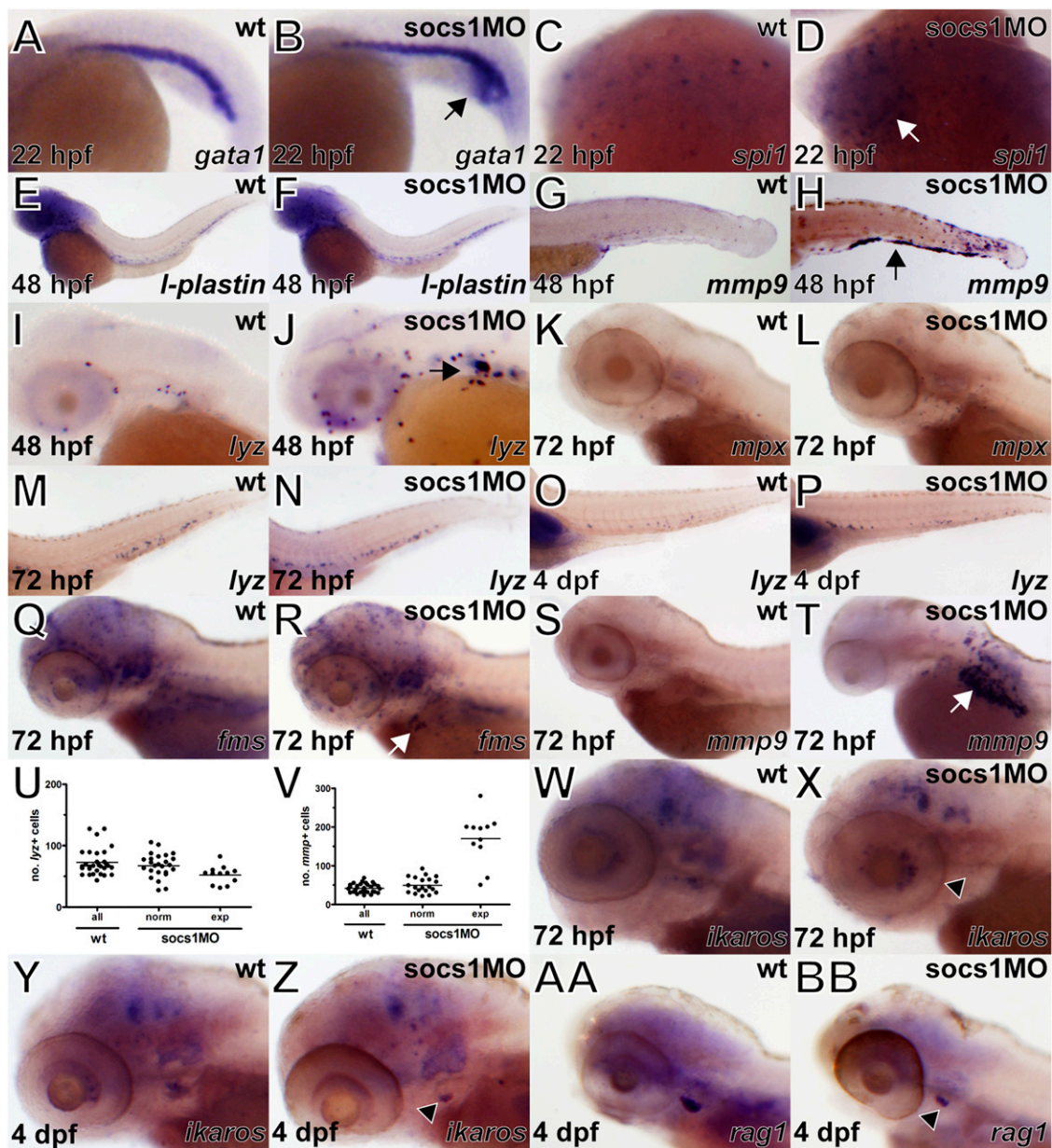
<sup>a</sup>Results are from a representative experiment in each case ( $n > 5$  experiments), with  $*p < 0.05$ .

extent of *ikaros* staining was reduced at 72 hpf (Fig. 4W, 4X), when lymphoid precursors are colonizing the presumptive thymus. Staining at 4 hpf with both *ikaros* (Fig. 4Y, 4Z) and *rag1* (Fig. 4AA, 4BB) was consistently reduced in around 50% of *socs1* morphants compared with controls.

#### Zebrafish *socs1* can interact with the Jak2–Stat5 pathway in vitro and in vivo

The enhanced early hematopoiesis observed in *socs1* morphants was consistent with our previous studies examining the in vivo effects of increased activation of zebrafish Jak2a (33, 54) or Stat5.1 (53), both of which are expressed in the pICM. Moreover, SOCS1 has been shown to be induced by receptors using the Jak2–Stat5 pathway (1). We therefore explored the interaction between SOCS1 and the Jak2–Stat5 pathway in zebrafish.

Analysis of the promoter of zebrafish *socs1* revealed the presence of a consensus TTCN<sub>3–5</sub>GAA Stat binding site (65) upstream of the transcription start site amid four GAAA units and a GC box (Fig. 5A), which are also present in the mouse *Socs1* promoter



**FIGURE 4.** *socs1* morphants exhibit perturbed hematopoiesis. A–Z, WISH of morphant embryos. Wild-type (diluent or SCR MO-injected) embryos (A, C, E, G, I, K, M, O, Q, S, W, Y, AA) or embryos injected with *socs1* MO (B, D, F, H, J, L, N, P, R, T, X, Z, BB) were analyzed by WISH at the time points indicated using the following specific hematopoietic lineage markers: erythroid cells, *gata1* at 22 hpf (A, B); early myeloid cells, *spi1* at 22 hpf (C, D); leukocytes, *l-plastin* at 48 hpf (E, F); *lyz* at 48 hpf (I, J), 72 hpf (M, N), and 96 hpf (O, P); heterophils, *mmp9* at 48 hpf (G, H) and 72 hpf (S, T); *mpx* at 72 hpf (K, L); macrophages, *fms* at 72 hpf (Q, R); lymphoid cells, *ikaros* at 72 hpf (W, X) and 4 dpf (Y, Z); and *rag1* at 4 dpf (AA, BB). All images are lateral view with anterior to the left. Changes in the extent of staining in injected embryos are indicated with arrows (increased) or arrowheads (decrease). U and V, Quantitation of myeloid cell numbers. Numbers of cells expressing *lyz* (U) or *mmp9* (V) at 72 hpf in SCR MO-injected embryos (wt all) or *socs1* MO-injected embryos differentiated on the basis of a normal (norm) or expanded (exp) pICM. Results are representative of four experiments.

(66). Nuclear extracts prepared from human 293T cells transiently transfected with zebrafish Stat5.1 and a hyperactive version of zebrafish Jak2a exhibited binding activity to the *socs1* promoter site at least as strong as that observed to the archetypic  $\beta$ -casein promoter element (67) (Fig. 5B), whereas binding could be competed out with unlabeled *socs1* or  $\beta$ -casein sites, confirming the specificity of binding (Fig. 5B). In contrast, extracts from cells transfected with empty vector or Stat5.1 or hyperactive Jak2a alone possessed no binding activity. Expression of *socs1* was reduced in the presence of a Stat5.1 MO (data not shown), suggesting that regulation of *socs1* by Jak2a/Stat5.1 was physiologically relevant.

The mammalian SOCS1 protein is known to negatively regulate Jak2 through high-affinity binding to the kinase activation loop via its SH2 domain and KIR, with the latter acting as a pseudosubstrate to further inhibit kinase activity, as well as downstream targets, including Stat5 (4, 5, 56). Alignment of the kinase activation loops of mouse Jak2 with those of the zebrafish Jak2a and Jak2b paralogs revealed very high conservation of this region, whereas homology between mouse and zebrafish SOCS in their respective KIRs was more modest (Fig. 5C). Importantly, however, the residues that were conserved correlated well to those previously identified as important in creating the pseudosubstrate motif that mediates Jak2 kinase inhibition (5), suggesting that the mech-





Zebrafish SOCS1 could inhibit the ability of mouse Gp130 to activate Stat3 (via Jak2) and of zebrafish jak2a to activate stat5.1 in vitro, consistent with conservation seen in the *socs1* KIR that is critical for inhibition of Jak2 kinase activity (5). The expression pattern of zebrafish *socs1* also overlapped to varying degrees with those of zebrafish *jak1*, *jak2a*, *jak2b*, and *stat3* (29, 30, 33, 70), as well as *stat5.1* (data not shown), consistent with a conserved functional interaction (7). Finally, like its murine equivalent (8, 66), the promoter region of the zebrafish *socs1* gene contained a consensus Stat site, which was able to be bound by zebrafish Stat5.1. Knockdown of the zebrafish *socs1* gene resulted in a reduced number of T cells in the thymus, consistent with studies in mice (17, 71). However, our data suggest that this lymphopenia might be due, at least initially, to reduced lymphoid precursors, rather than hyperresponsiveness to lymphopoietic cytokines such as IL-7 and IL-15 (72, 73). In addition, expansion of primitive hematopoietic progenitors and disruption of both myelopoiesis and erythropoiesis were observed, consistent with the increased macrophage progenitors, mild neutrophilia, and altered hematocrit observed in *Socs1*-knockout mice (74). Collectively, these results suggest that zebrafish represents a worthwhile model for the further characterization of *socs1* function with relevance for mammalian systems.

Indeed, some clear new insights were apparent from this work. Thus, the phenotypes observed in mice were thought to be largely due to hypersensitivity of T cells to IFN- $\gamma$ , exacerbated by an increased production of this cytokine, leading to a sustained inflammatory response (10, 16). However, the ontogeny of development in zebrafish means that the myeloid phenotypes observed in *socs1*-morphants occur a considerable time before T cells are formed (27), providing definitive proof that the early effects on myeloid cells are T cell independent. Such a direct effect on myeloid cells is supported by the expression of *socs1* within the pICM, a site of primitive myelopoiesis. This notion is consistent with other studies showing a direct effect of SOCS1 in hematopoietic disease. For example, inactivation of the *SOCS1* gene (via methylation) is observed in acute myeloid leukemia (75) and chronic myeloid leukemia (76), whereas other studies have identified SOCS1 as a key player in various inflammatory diseases including rheumatoid arthritis (77–79). It is also concordant with a recent study showing that T cell/NKT cell-specific depletion of *Socs1* is insufficient to cause inflammatory disease (13). Hypersensitivity to IFN- $\gamma$  could still be important for these T cell-independent effects. Indeed, macrophage sensitivity to IFN- $\gamma$  is significantly increased in *Socs1*-deficient mice (80). SOCS1 regulates IFN- $\gamma$  at least in part via recruitment to the IFN- $\gamma$ R (12). However, it can also regulate IFN- $\alpha$  $\beta$ R, by direct binding (24) or via a direct effect on Tyk2. Importantly, all of these signaling pathways are conserved in fish.

A major outstanding question from this work is exactly which receptors are regulated by SOCS1 to mediate its effects on hematopoietic development independently of T cells. We demonstrate that zebrafish SOCS1 is able to act as a negative regulator of the Jak2–Stat5 pathway both in vitro and in vivo, which suggests that receptors using this pathway are involved. In support of this hypothesis, expression of hyperactive jak2a (33) or constitutively active stat5.1 (53) in zebrafish produced similar phenotypes to those observed in the *socs1* morphants, including increased WBC numbers and a red cell defect. The Jak2–Stat5 pathway lies downstream of several cytokine receptors, including G-CSFR, IL-3R $\beta_c$ , and EpoR, all of which have clear zebrafish homologs (32, 34). Similarly, G-CSFR controls the development of several myelomonocytic populations in zebrafish (37), with SOCS1 known to control Stat5 activation mediated by this receptor (81).

Others have shown murine *Socs1*<sup>-/-</sup> hematopoietic progenitors to be hyperresponsive to GM-CSF, which uses IL-3R $\beta_c$  (74). These observations suggest uncontrolled signaling by G-CSF and/or GM-CSF may contribute to the myeloid phenotypes observed in SOCS1 morphants. Alternatively, Gp130 is the common signal transducing chain for a number of important cytokine receptors, including IL-6R (82), with orthologs for both Gp130 and IL-6R $\alpha$  also found in zebrafish (32). Gp130 is known to have a prominent role in hematopoiesis (83, 84) and is able to be inhibited by SOCS1 (56), an activity we showed is conserved in the zebrafish protein. Therefore, aberrant Gp130 signaling in *socs1* morphants, particularly via the IL-6R complex, may contribute to the phenotypes observed, including the hematopoietic progenitor defects, albeit likely that this is via Stat3 rather than Stat5. Finally, the EpoR has been shown to be intimately associated with RBC production (85), including in zebrafish (34), whereas *Socs1* is induced in response to Epo and can negatively regulate EpoR-mediated signaling through the Jak2–Stat5 pathway (7, 86). Therefore, dysregulated EpoR signaling may explain the perturbed erythropoiesis in *socs1* morphants.

It is worthwhile, however, to consider other possibilities. SOCS1 is known to mediate negative-feedback regulation of LPS-induced TLR4 signaling in macrophages via several potential mechanisms. Stimulation of macrophages with LPS is able to induce SOCS1 (87, 88) that can regulate MyD88-dependent TLR4 signaling by direct degradation of the adaptor Mal (26) or via destabilization of p65/RelA (89, 90), thereby decreasing production of proinflammatory cytokines such as IL-6 and TNF- $\alpha$ . It can also limit MyD88-independent TLR4 signaling by suppressing the paracrine actions of IFN- $\beta$  induced through this pathway via its actions on Tyk2 (25). Indeed, it appears able to decrease sensitivity to other cytokines, including IFN- $\gamma$ , IL-6, or GM-CSF, suggesting a wider effect (91, 92). Loss of SOCS1 leads to LPS hypersensitivity, with prolonged NF- $\kappa$ B, increased production of proinflammatory cytokines, and increased sensitivity to their actions (22). Clearly, such a mechanism may also contribute to the increased numbers myeloid cells observed in *socs1* morphants via induction of an inflammatory response, because embryos are chronically exposed to LPS through normal animal husbandry. The zebrafish is well poised as an experimental model to shed further insight into the exact mechanism(s) involved.

## Acknowledgments

We thank Ben Kiefel, Sarah Stephenson, and Simon Yoong for help and advice and Daniel Merlo and staff at the Deakin Animal House for invaluable technical support.

## Disclosures

The authors have no financial conflicts of interest.

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