**csrnp1a** Is Necessary for the Development of Primitive Hematopoiesis Progenitors in Zebrafish

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**Abstract**

The CSRNP (cystein-serine-rich nuclear protein) transcription factors are conserved from *Drosophila* to human. Functional studies in mice, through knockout for each of their paralogs, have resulted insufficient to elucidate the function of this family of proteins in vertebrate development. Previously, we described the function of the zebrafish ortholog, Csnrp1/Auxd1, showing its essential role in the survival and proliferation of cephalic progenitors. To extend our understanding of this family, we have studied the function of its paralog *csrnp1a*. Our results show that *csrnp1a* is expressed from 0 hpf, until larval stages, particularly in cephalic territories and in the intermediate cell mass (ICM). Using morpholinos in wild type and transgenic lines we observed that Csrnp1a knockdown generates a mild reduction in head size and a depletion of blood cells in circulation. This was combined with *in situ* hybridizations to analyze the expression of different mesodermal and primitive hematopoiesis markers. Morphant embryos have impaired blood formation without disruption of mesoderm specification, angiogenesis or heart development. The reduction of circulating blood cells occurs at the hematopoietic progenitor level, affecting both the erythroid and myeloid lineages. In addition, cell proliferation was also altered in hematopoietic anterior sites, specifically in spi1 expression domain. These and previous observations suggest an important role of Csnrps transcription factors in progenitor biology, both in the neural and hematopoietic lineages.

**Citation:** Espina J, Feijóo CG, Solís C, Glavic A (2013) csrnp1a Is Necessary for the Development of Primitive Hematopoiesis Progenitors in Zebrafish. PLoS ONE 8(1): e53858. doi:10.1371/journal.pone.0053858

**Editor:** Michael Klymkowsky, University of Colorado, United States of America

**Received:** May 4, 2012; **Accepted** December 4, 2012; **Published** January 9, 2013

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**Funding:** Funding was provided by FONDAP grant 15090007 (A.G.), Universidad Nacional Andres Bello grant DI-07-09/R (C.G.F. and A.G.) and FONDECYT grant 11090102 (C.G.F.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

All vertebrates, including teleosts, have two waves of hematopoiesis occurring sequentially during development. The first is the so-called primitive hematopoiesis, which produces mainly erythrocytes and primitive macrophages. The second is the definitive hematopoiesis, which generates long-term hematopoietic stem cells (HSC) capable of unlimited self-renewal and which is able to generate all mature hematopoietic lineages. In zebrafish, primitive hematopoietic cells arise from two distinct territories of the lateral plate mesoderm (LPM), anterior and posterior, which can be evidenced by the expression of the early hematopoietic marker tal1/scl (T-cell acute lymphocytic leukemia 1) [1]. The anterior LPM gives rise to the rostral blood island (RBI), while hematopoietic cells from the posterior LPM migrate ventrally towards the midline to fuse, forming the intermediate cell mass (ICM) [2]. The RBI generates macrophages and endothelial cells whereas cells from the ICM differentiate as endothelial cells of the trunk vasculature, neutrophils, and proerythroblasts. The primitive erythroblast population arises from a subset of posterior tal1 expressing cells that also express the Krüppel-like transcription factor klf4 [3] and the zinc finger transcription factor gata1 [4]. These proteins are expressed bilaterally in the posterior LPM and promote the expression of genes essential for erythroid differentiation like hbb1 [5]. On the other hand, from a subset of anterior tal1/scl expressing cells, which also express spi1, mature macrophages and neutrophils arise, which are recognized by the expression of markers like lymphocyte cytosolic plastin1 *lep1*, and myeloid-specific peroxidase *mpx* respectively [6].

The CSRNP family of transcription factors has been conserved from *Drosophila* to humans. These proteins do not have any recognized domain or structural motif recorded previously in any database. However, *in silico* analysis has identified a motif of three regions at the amino terminus clearly present in every member of this family. The first region is rich in serine followed by a basic domain and a final cysteine-rich region [7]. The subcellular localization of *csrnp* gene products, in the three mouse paralogs, CSRNP-1, 2 and 3, as well as in the single *Drosophila* ortholog described, DAxud1, is the nucleus [7], [8]. This, together with the fact that the mouse protein can activate transcription in a Gal4 fusion assay [7] suggests that these proteins have transcription factor characteristics and might behave as such. The in vivo function of these proteins has been studied by obtaining single knockout mice for each of the three paralogs and successively through combinations of double and triple knockout animals. The mutant mice were indistinguishable from wild-type in every aspect analyzed with the exception of the triple KO mice, which died neonatally [7]. In *Drosophila*, there is only one member of the CSRNP family, DAxud1. Functional studies revealed that DAxud1 regulates distinct aspects of the cell cycle and apoptosis, suggesting that it could behave as a tumor suppressor [8]. Its
overexpression causes, through changes in CDK1 activity, a G2/M cell-cycle arrest, and apoptosis induction through the activation of the JNK pathway; in addition DAxud1 knockdown increases the proliferation of imaginal disc cells [9]. This last result is in correspondence to previous publications showing that human AXUD1 message decrease in cancers associated with mutations in the Wnt pathway transducer AXIN1 [9]. Moreover, it has been found that DAxud1 interacts genetically with Sp65 [10]. [Glieve unpublished results]. The gpl5 product together with Sp6, are part of the transcription elongation factor DSIF. In zebrafish this elongation complex, and in particular Sp6/Foggy, regulate erythropoiesis through gata1 expression [11].

Two sequences homologous to DAxud1 have been identified in zebrafish, with 69% and 63% of amino acidic identity [12]. Previously, we reported the functional analysis of the closest sequence to the Drosophila DAxud1 present in zebrafish, the Csrnp1/Axud1 gene, showing that it is essential for the expansion and survival of cephalic neural progenitors [12]. Here, we present the expression and functional analysis of the second zebrafish paralog, csrnp1a. Our expression studies showed that csrnp1a is present from cleavage stages and, later on, its transcription becomes dynamically restricted to two territories, the cephalic domain and the caudal region, specifically at the ICM. Knockdown analysis indicated that csrnp1a morphants display a mild head size reductions and a remarkable decrease in circulating blood cells. This phenotype is probably the result of a decrease in the erythroid transcription factor gata1, a change of the expression in the gpl1 signal at 12 somites and a diminished cell proliferation in gpl territories, all of which are indicative of a role for csrnp1a in the development of primitive blood lineages. Together, these results expand our understanding about the role of the CSRNP protein family in vertebrate development.

Results

csrnp1a Expression Pattern

In previous work, our laboratory identified the zebrafish csrnp1 gene and characterized its function [12]. During the analysis of related sequences, a second member of the Csrnp family, Csrnp1a, was also identified (accession number: XM_683666.4). This protein of 514 amino acids has 63% identity at amino acid level to the Drosophila AXUD1 and its coding sequence maps to chromosome 2 of zebrafish at locus NC_007113. The gene spans 14.2 kb, and consists of 5 exons that generate a predicted messenger of 2941 bp (Fig. S1).

To identify the temporal expression pattern of csrnp1a we performed RT-PCR using cDNA templates obtained from embryos at 0, 10, 18, 24, 30, 36 and 48 hours post fertilization (hpf) (Fig. 1M). This showed that csrnp1a is maternally inherited and expressed through all the time points analyzed. To obtain its spatial expression pattern, we performed in situ hybridizations at different stages. At early stages, csrnp1a is distributed over the entire embryo (Fig. 1A, B, C). Later on, at 19 hpf, its expression begins to be restricted to ventral regions along the A-P axis (Fig. 1D). Between 24 and 36 hpf, it is possible to detect its ventral expression in anterior cephalic tissues and in posterior regions (Fig. 1E to I, black and red arrows respectively). In the anterior region, the expression is initially ubiquitous (Fig. 1C, D) and then limited to the ventral domain (Fig. 1E, G, I, black arrows). The posterior expression is progressively circumscribed to the intermediate cell mass (ICM), the primary hematopoietic tissue in zebrafish. In this territory, the expression of csrnp1a is very dynamic. At 24 hpf, an initial extension is detected in the ICM (Fig. 1E, F), which reaches its maximum at 30 hpf (Fig. 1G, H). Later, at 36 hpf, the distribution of mRNA becomes gradually restricted (Fig. 1I, J), disappearing at around 40 hpf (Fig. 1K, L). The comparison between csrnp1 and csrnp1a reveals that although both genes are expressed in cephalic domains, paralog specific expression territories can be assigned. Particularly we detected csrnp1a transcription in the ICM at the time when hematopoietic progenitors expand and the initial blood cells reach circulation.

This observation, together with the fact that the ICM is a primary site of proliferation and differentiation of hematopoietic cells [13], has lead us to postulate a role for csrnp1a in developing hematopoietic cells.

Knockdown of csrnp1a in Developing Embryos

To test the aforementioned hypothesis, we injected morpholino oligonucleotides (MOs) designed to suppress the expression of csrnp1a. To avoid or reduce the nonspecific effects of MO injection we examined two different types of morpholinos, one designed to bind to the ATG site (Mocsrnp1a atg), thus inhibiting translation, and the other designed to block the correct splicing of the primary message (Mocsrnp1a sp5) through its binding to the donor and acceptor sites at the second and third exons (Fig. S1). To confirm the efficiency of this last morpholino and to test if the morpholino is acting in a dose-dependent manner, we injected the splicing morpholino at 2.5 and 5 ng per embryo, showing by qPCR (Fig. 2I) and RT-PCR (Fig. S2) the dose-dependence reduction at expression at 24 hpf. In addition we checked the decrease of the csrnp1a RT-PCR signal in embryos at 30 hpf, observing that csrnp1a processed messenger reaccumulate (Fig. S2). Thus this splicing morpholino was no longer effective at this stage. The phenotype resultant of morpholino activity and therefore informative about the role of csrnp1a, was a mild head reduction and the notable lack of circulating cells in embryos at 26 hpf. The concentration of morpholino used to achieve these phenotypes was 2.5 and 5 ng per embryo for the ATG and splicing morpholino respectively. This phenotype was generated by both morpholinos in 34% of the injected embryos (Fig. 2A, B, C and quantified in H), suggesting that both morpholinos specifically knocked down csrnp1a. The percentage of embryos with the blood phenotype was related with the amount of splicing morpholino injected, being the 5 ng the maximum tolerated before massive embryo death (Fig. 2H). Although we have tried in several opportunities and with different strategies to clone the full length csrnp1a, we have not being able succeeded, so the ideal rescue experiment to confirm this assumption is impossible to be carried out at this moment.

On the other hand, to better visualize the reduction of blood cells observed in morphant embryos we used two different stains: O-dianisidine, which reacts with the hemoglobin present in erythrocytes generating an orange precipitate [14] and Diaminobenzidine (DAB), which detects the myeloperoxidase activity in myeloid cells [15], although using longer development of this stain also labels the intermediate cells mass. A significant decrease in both stains was obtained in morphant embryos at 33 hpf, corroborating the in vivo observation of reduced blood cells in circulation (Fig. 2A, B, C, F and G). Conversely, and in agreement with the observed reduction of morpholino efficiency at 30 hpf (Fig. S2), we detected a reversion of the blood phenotype in 77.3% of the morphant embryos at 48 hpf (Fig. 2E). Taken together, these results suggest that both myeloid and erythroid lineages are diminished in the csrnp1a knockdown condition.

Csrnp1a Reduction does not affect csrnp1 Expression

Due to both members of the zebrafish Csrnp family share expression territories, we decided to test if there is any regulation between Csrnp1a and csrnp1. To perform this analysis we injected the csrnp1a splicing morpholino and analyzed csrnp1 expression by
in situ hybridization at two stages, 8 somites and 30 hpf. We did not find any difference in csrnp1 expression between control and csrnp1a morphants (data not shown). This observation was corroborated by RT-PCR of csrnp1 in csrnp1a morphant embryos (Fig. 3A). Likewise, we hypothesized that matching expression territories of Csnrp1 and Csnrp1a at early stages of development could reflect similar functions. To test this we co-injected the csrnp1a splicing morpholino with the csrnp1 atg morpholino [12]. As can be observed in Figure 3, csrnp1a knockdown generated the blood cell decrease previously described in 38.7% of the injected
embryos (Fig. 3C). Similarly, the csrnp1 morpholino also generated the reduction in blood cells but only in 22.6% of the injected embryos (Fig. 3D). Importantly, the amount of embryos with the blood phenotype was enhanced up to 64.4% when both morpholino were co-injected (Fig. 3E). This result suggests that both genes may have redundant or complementary functions in the hematopoietic process.

csrnp1a does Not Participate in Early Mesoderm Patterning

To gain further insight about this phenotype and the specific role of csrnp1a on its generation, we decided to examine the earliest stage of hematopoiesis affected in csrnp1a morphants. Since hematopoietic cells arise from a distinct mesodermal domain, we first analyzed whether the early specification or patterning of this territory was affected. The expression of the specific mesodermal markers goosecoid, notail and gata5 indicated that there were no differences between morphant and control embryos (Fig. S2), excluding the possibility that alterations in mesoderm specification or patterning were responsible for the phenotype described. Next, we examined whether the selection of early progenitors was disturbed in morphant embryos. To this end we analyzed the expression of the earliest hematopoietic marker in zebrafish, T-cell acute lymphocytic leukemia 1, tal1, which is expressed in the primitive hematopoietic stem cells and vascular precursors [16]. At 8 somites (13 hpf), approximately 5 hours after progenitor selection, no substantial differences in the expression of this gene was seen between control and morphant embryos (Fig. 4A to D), suggesting that early progenitors of hematopoietic cells were correctly specified.

![Fig. 2. csrnp1a knockdown decreases the number of blood cells in circulation. O-Dianisidine stain labels erythrocytes (A, B, C, D, E) and Diaminobenzidine (DAB) blood cells in general at the ICM (F and G). The injection of csrnp1a morpholino produces a dramatic reduction of blood cells in 34% of the injected embryos (n = 16/47) at 33 hpf (A, B, C, F, G) which is reverted at 48 hpf (D, E) (all are lateral views, anterior to the left). (H) Chart summarizing the morpholino dose-phenotype quantifications of 26 hpf control embryos (n = 71) and injected with 2.5 ng (n = 69), 5 ng (n = 85) or 10 ng (n = 87) of Mocsrnp1a spt morpholino. (I) qPCR with primers located in exon 2 and 3 of 24 hpf control embryos (C) and splicing morphants injected with two concentrations, 2.5 and 5 ng per embryo, showing the relative expression of csrnp1a message, which decreases significantly in a dose-dependent manner (t student p<0.01).

doi:10.1371/journal.pone.0053858.g002

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PLOS ONE | www.plosone.org 4 January 2013 | Volume 8 | Issue 1 | e53858
In accordance to this, we observed that blood vessels marked with GFP under the promoter of the endothelial marker fli1a, in the transgenic line Tg(fli1a:EGFP)y1, which develops from subsets of tal1 positive cells, were correctly formed (Fig. 4E and F). This last result also excludes the possibility that defects in blood circulation arise as a consequence of vessel malformations. Likewise, another factor that could explain the decrease in circulating cells is an extremely reduced blood flow [17]. This possibility was excluded by two indirect approaches. Nitric oxide (NO) is a well-established direct regulator of vascular tone and reactivity, thereby influencing blood flow [17], so we incubated morphant embryos in the NO donor S-nitroso-n-acetyl-penicilamine (SNAP), and tested whether this compound was capable to rescue the morphant phenotype enhancing blood flow.

csrnp1a knockdown phenotype was not rescue by SNAP treatment (Fig. S3). Second, in situ hybridizations using auricular (amhc) and ventricular (vmhc) probes were used to check for heart malformations that could influence blood flow. Signal of both amhc and vmhc in morphant and controls embryos were indistinguishable between each other (Fig. S3). Additionally, the number of heartbeats was determined in order to discard heart failure as a source of reduced blood circulation. We detected a marginal, whilst significant, heartbeat reduction in morphant embryos (Fig. S3), but we believe this reduction is too small to generate the observed phenotype. This last result supports the notion that blood circulation defects do not arise as a consequence of heart malformation. Together, these results suggest that a decrease in blood flow is not the cause of the csrnp1a morphant phenotype.

**Csrm1a Is Essential during Erythropoietic Lineage Development**

In order to establish if csrm1a function is necessary in the erythropoietic branch of hematopoiesis, we evaluated the expression of klf4 and gata1 by in situ hybridization. These transcription factors are expressed in the posterior lateral plate mesoderm acting downstream tal1 in the specification of the erythroid lineage. Also, klf4 directly activates gata1 and thus both genes are expressed in the posterior lateral plate mesoderm [3].

At 8 somites, no significant differences in the expression of klf4 were detected between control and morphant embryos (Fig. 5A, B), suggesting that erythroid precursors were correctly specified at this stage in the differentiation process. In contrast, morphant embryos showed a strong reduction of gata1 expression (Fig. 5C, D), indicating that csrm1a regulates gata1 expression and erythropoietic development downstream klf4. This inhibition was not the result of a general developmental delay as revealed by the invariable pattern of myoD stripes between morphant and control embryos (Fig. 5C, D). Accordingly, we observed that gata1 inhibition was accompanied by a decline in the amount of mature erythrocytes, which was verified by the diminished expression of embryonic alpha hemoglobin 1, hbae1 (Fig. 5E, F).

Considering the described role of Csrnp1 in cephalic survival we decided to investigate if the restriction in gata1 expression and the decrease in mature erythrocytes were due to cell death induction or perturbations in proliferation. Robu et al. (2007) reported that morpholino injections increase cell death activating the p53 pathway [18]. Consequently, we decided to co-inject p53 with csrm1a morpholinos and compare the number of cells labeled with...
Figure 4. Csrnp1a reduction does not affect the hemato-angiogenic mesoderm. In situ hybridization indicating the expression of the hemangioblast marker, tal1/scl at 8 somites (A to D) (A, B anterior view; C, D posterior view). Control and morphant 32 hpf transgenic zebrafish embryos expressing GFP under the promoter of the endothelial marker fli1a (Tg(fli1a:EGFP)y1)(E, F) (lateral view, anterior to the left). We did not observe difference in either marker in 100% of the injected embryos (n = 53).
doi:10.1371/journal.pone.0053858.g004

Figure 5. Erythroid/Gata1 precursors are affected by csrnp1a knockdown. (A, C, E) Control and (B, D, F) morphant embryos. At 8 somite stage the expression of the erythroid marker klf4 was not affected in morphant embryos (A, B) (100%, n = 48). However a clear decrease in gata1 expression was detected in csrnp1a deficient embryos (compare C with D) (54.8%; n = 23/42) without major changes in the number of myoD stripes (7.1%; n = 3/42, more than 2 stripes), which served as readout of developmental progression. Control embryos showed normal gata1 expression in 100% of the cases (n = 0/27) and 3.7% have altered myoD expression (n = 1/27). In agreement with gata1 inhibition, at 32 hpf terminal differentiation marker, hbae1, was reduced in morphant embryos (E, F) (32.6%; n = 14/43).
doi:10.1371/journal.pone.0053858.g005
the cell death marker, acridine orange. At 8 somites, morphant embryos displayed a generalized increase in cell death that dropped to control levels when co-injected with p53 morpholo.

Importantly, the suppression of cell death produced by p53 and csrnp1a morpholo co-injection did not modify the reduction of blood cell in circulation or the phenotype generated by Csrnp1a knockdown (Fig. S4). At this same stage we also checked proliferation by immunohistochemistry against H3-P but no significant change was detected in the tall expressing area (data not shown).

Csrnp1a is Necessary for Myeloid Lineage Formation

Finally, in attention to the reduction in DAB stain observed in the ICM in morphant embryos, we tested whether csrnp1a could also participate in the differentiation of the myeloid lineage. The transcription factor spi1 was reported to be an early key factor in the process of primitive mielopoiesis in zebrafish [6]. In situ hybridization assay showed that csrnp1a knockdown modifies spi1 expression pattern, not by decreasing spi1 signal but changing its spatial distribution (Fig. 6A to D). At 12 somites, it is possible to observe a scattering of the spi1 expression domain in csrnp1a knockdown embryos, while in control embryos this signal is compact. On the other hand, when cell proliferation was examined, a significant decrease in the number of Phosphorylated-Histone H3 positive cells in the spi1 expression domain was detected (Fig. 6E to G). These results imply that Csrnp1a knockdown affects also the myeloid branch of primitive hematopoiesis. The effect on early myeloid progenitors was verified by the observation that the number of differentiated myeloid cells, neutrophils and macrophages, drastically decrease in morphant embryos marked with the lpl probe and in transgenic morphant embryos expressing GFP under the myeloperoxidase promoter (Tg(Bacmpx:GFP)i114) (Fig. 6H to K).

Discussion

csrnp1a Expression during Early Zebrafish Development

The expression pattern of csrnp1a is highly dynamic, showing an initially ubiquitous distribution, and then localizing over the ventral side of the embryo, expanding and retracting over the ICM. When we compared this expression with its paralog [12] we can see that csrnp1a and csrnp1 are not identically expressed. However both genes share expression in anterior regions of the neural tube, suggesting that common regulatory mechanisms, perhaps Shh signaling as in the case for csrnp1, operate in this territory to control csrnp1a. Additionally, csrnp1a is transcribed in the intermediate cell mass, which is the site of proliferation and differentiation of primitive hematopoietic cells [13]. Zebrafish has two waves of hematopoiesis, the primitive and definitive. However considering that reductions of blood cells were observed between 26 and 36 hpf, specifically of cells arising from the primitive hematopoietic process, and the absence of appropriate tools to study the definitive wave, is that we focused in the effect of csrnp1a in primitive hematopoiesis. Finally, the expression domains described for both paralogs support the notion that the Csrnp family could have common roles in the biology of progenitor cells in the nervous system as well as in hematopoietic precursors. This suggestion is further sustained by our functional analysis.

Csrnp1a Function during Early Zebrafish Development

At 30 hours post fertilization the phenotype of csrnp1a morphant embryos included a severe decrease in circulating blood cells, that is consistent with its expression in the ICM. This is reverted at 48 hpf, which is consistent with the observation that at 30 hpf the splicing morpholino ceases its function. This reversal could be explained because hematopoietic progenitors were not completely depleted or failed to differentiate and get arrested in an immature stage, and then when csrnp1a is processed again, the progenitors expands, differentiation resumes and the cells eventually go into circulation.

In addition, a generalized increase in cell death was observed in csrnp1a morphants, though this was considered nonspecific since co-injection with the p53 morpholo suppresses this effect. Importantly, when these embryos were screened at 32 hpf for reductions in blood cells in circulation, the proportion of embryos displaying the phenotype remained the same. This indicates that cell death is likely a consequence of morpholino toxicity and has no impact on the hematopoietic phenotype described.

In this study, we showed that Csrnp1a knockdown generates a developmental perturbation in the erythroid and myeloid primitive blood lineages, affecting gata1 transcription and spi1 message distribution respectively, however without effects on upstream genes like klf4 or tall1.

Csrnp1 and Csrnp1a Relationship

At early stages csrnp1 and csrnp1a share expression domains suggesting a probable cross regulation or redundant functions. Csrnp1a knockdown does not impair csrnp1 expression indicating that csrnp1 is not a target of Csrnp1a, however, csrnp1 morphants present the blood phenotype in 22.6% of the injected embryos. Since csrnp1 is not expressed in the ICM at later stages, as is csrnp1a, this phenotype should derive from its early function. At 8 somites csrnp1 is expressed covering the lateral plate mesoderm, where the hematopoietic progenitors arise [12]. In accordance to this, when csrnp1a and csrnp1 morpholinos are co-injected the number of embryos presenting the blood phenotype increase up to 64.4%, pointing to a redundant role of both csrnp genes in hematopoiesis. This result and the fact that csrnp1 acts at 8 somites strength the notion that csrnp1 is also exerting its hematopoietic function at these early stages.

Erythropoietic Function of csrnp1a

In zebrafish, the first blood progenitors are specified in mesoderm during gastrulation. BMP4 first induces ventral-posterior mesoderm and subsequently directs a subpopulation of these cells towards the blood fate by activating snta3a, eda and hox genes [19]. These regulatory interactions induce the transcription factor, tall/scl, which is central for the specification of hematopoietic precursors.

tall/scl is expressed in hemangiblast, undifferentiated cells capable to produce blood and vascular lineages [16]. This protein form complexes with Lmo2, Gata2 and Gata1, controlling the expression of a variety of hematopoietic genes [20]. Downstream of these are discriminating factors essential for erythroid and myeloid development, as gata1 and spi1 respectively. Knüppel-like, klf4, is another important transcription factor that promotes gata1 activation by direct binding to its promoter [3]. Our observations indicate that the observed inhibition of gata1 in Csrnp1a morphants is not caused by deficiencies in klf4 expression. Furthermore, the differential defects produced over gata1 and spi1 expression at the 8–12 somite stage, strongly suggests that at this time Csrnp1a could be part of a Tall/Scl complex implicated in erythroid differentiation, specifically regulating gata1 transcription.

It has been shown that gata1 is also controlled by the transcription elongation factor DSIF. The spt5/Foggy mutant revealed that DSIF complex promotes gata1 transcription [11]. It has been speculated that DSIF could recruit P-TEFb conferring
functional specificity. P-TEFb also interacts with hematopoietic transcription factors such as Tif1-γ, LDB1, and Gata1, which are components of the Scl complex [11]. The observation that Drosophila Axu1 interacts in a double hybrid assay [10] and genetically with Spt5 (Glavic, unpublished result) reinforces the notion that Csrnp1a could be part of the Scl complexes involved in erythroid differentiation and gata1 expression.

Myelopoietic Function of Csrnp1a

The transcription factor spi1 is a key factor in the myeloid lineage. Our results show that Csrnp1a knockdown produces a distortion in its expression pattern, which does not seem to be solely due to transcriptional inhibition. The Scl complex in anterior lateral plate mesoderm regulates the myeloid specific factor spi1; here the specification of spi1+ cells relies on the BMP pathway [21]. Another factor acting on spi1 is retinoic acid. Embryos exposed to it show a decreased expression of tal1, lmo, gata2, and eprs in anterior hemangioblasts. Finally, and analogously to the erythroid lineage decisions, these factors drive the differentiation of anterior LPM cells to neutrophils and macrophages inducing the expression of markers like mpo or lcp [22].

The actual model of cell fate decisions between myeloid and erythroid lineages involves a cross negative regulation between spi1 and gata1 [22]. Recent data has added an extra layer to this regulation, the tif1-γ transcription factor, which according to the context can repress or activate spi1 and gata1. At early stages tif1-γ morphants show a decrease in the expression of both genes [23].

Our results indicate that Csrnp1a influences, at different stages and independently, the development of erythroid and myeloid lineages without affecting the common progenitor revealed by tal1 expression. A potential explanation to this arises considering developmental time: the fact that tif1-γ is capable to decrease both factors at early stages and that csrnp1a interacts with Spt5, a member of the DSIF complex [10], [Glavic unpublished results].

Figure 6. Myeloid lineage is affected by csrnp1a knockdown. Morphants embryos show an alteration in the spi1 signal (A to D; A, B lateral views anterior to left; and D, E anterior views). 85.7% of control embryos exhibit normal spi1 expression pattern (n = 42/49) (A, C), while csrnp1a morphants exhibit altered spi1 expression in 59.2% (n = 61/103) (B, D). The spi1 changes are accompanied by a significant decrease in proliferation (showed as the number of H3P within the spi1 signal area) (E, F, and G). Finally, a decrease in the macrophage specific marker lcp1 (43.8%; n = 14/32) is evidenced by ISH (H, I), and a clear reduction in the neutrophil specific marker mpo is evident in the transgenic line expressing GFP under the mpo promoter Tg(Bacmpo;GFP)114 (J, K) (47.2%; n = 25/53). All are lateral views, anterior to the left.

doi:10.1371/journal.pone.0053858.g006
DSIF interacts with the positive (P-TEFb) and negative elongation factors (NELF) to exert its function, and on the other hand P-TEFb binds to Tgf1-γ and the Tal1/Scl complex to regulate erythroid development [24]. Although DSIF has not been implicated yet in early myeloid, the abovementioned data let us speculate that Csrnp1a, and possibly Tgf1-γ, control over sp5 transcription and therefore the clear reduction in circulating blood cells probably arises as a specific role in primitive hematopoiesis. 

As mentioned before, Csrnp1a did not affect tal1 expression, and accordingly no alterations in endothelial markers or vessels morphology were detected. In addition, knocking down this gene did not disturb heart development, however we found a slight reduction in the heartbeat in morphant embryos. We consider this reduction too small to account for the very significant decrease in circulating blood cells. This is supported by the fact that blood cell flow density (number of cells in circulation moving through the visual field in a certain period of time) is almost null in morphant embryos, indicating that the problem is not based on blood flow but rather on the quantity of cells in circulation. Taken together all these data suggest that Csrnp1a has no influence in blood flow, and therefore the clear reduction in circulating blood cells probably arises as a specific role in primitive hematopoiesis.

In this paper we described new data regarding the Csrnp protein family in vertebrate development, specifically investigating zebrafish Csrnp1a. We have established a new role for this transcription factor family in primitive hematopoiesis. Further experiments are needed to precisely define the role of Csrnp1a in the transcriptional network controlling it. Special attention requires the relation of Csrnp1a with Spt5 and the formation and activity of selective Scl complexes during erythroid and myeloid differentiation.

Materials and Methods

Ethics Statement

All animals subjected to experimentation were anesthetized and procedures complied with the guidelines of the Animal Ethics Committees of the Universidad Andres Bello and Universidad de Chile, which approved this study.

Zebrafish Maintenance

Zebrafish were maintained and raised in our facility according to standard protocols [25]. The following fish strains were used in this study: Tab5 (wild type), Tg(Bacmpx:GFP)i114 [27]. All embryos were collected by natural spawning, staged according to Kimmel et al. 1995 [28], and raised at 28.5°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, without methylene blue, equilibrated to pH 7.0) in Petri dishes, as described previously [25]. Embryonic and larval ages are expressed in hours post fertilization (hpf).

Embryos Micro-injections and S-nitroso-N-acetylpenicillamine (SNAP) Incubation

Injections were carried out on 1- to 2-cell stage embryos. To repress csrnp1a gene expression, two morpholinos were designed (Gene Tools), an ATG-targeting (csrnp1a atg) 5'-ACTGAGGACCAACTCAAGAGCAG-3' to impair csrnp1a translation and a splicing morpholino (Mcsrnp1a sp1) 5'-TATCATGAGTGACCTTTGCGATG-3' targeting the exon 2/intron 2 splice site. Mcsrnp1a atg was used at 2.5 ng per embryo and Mcsrnp1a sp1 at 5 ng per embryo. Both morpholinos were diluted in distilled water. To test the genetic redundancy between csrnp1a and csmp1a we used a csmp1 atg morpholino at the concentrations previously described [12]. Also we used a control morpholino (5'-CAATCTTCATCTCTCAGGATGTCGAGG-3') [29] injected at 2.5 and 5 ng. To check the effectiveness of Mcsrnp1a sp1 we observed the decrease of Csrnp1a mRNA through RT-PCR using cDNA made from splicing morphants and compared with control embryos. The p33 morpholino (5'-GCCGCATTTGGCTTTGCAGAGATTTG-3') was injected at the concentration described by Robu et al. 2007 [18].

Zebrafish embryos were incubated in 10 μM of SNAP between 10 somites and 23 hpf in E3 medium. Dimethyl sulfoxide (DMSO) carrier content was 0.1%.

RT-PCR and qPCR

Total RNA was extracted from embryos at 0, 10, 19, 24, 30, 36, 40, 50 hpf. 50 embryos of any of these stages were homogenized in TRIzol solution (Invitrogen) following manufacturer's instructions and cDNA generated using Superscript II reverse transcriptase (Invitrogen) and oligo dT (Invitrogen) following also manufacturer's instructions. PCR was conducted on resulting cDNA using the primers: Csrnp1aF: 5'-CTCTGAGTGAAGACAGTCCACACAAG-3' and Csrnp1aR: 5'-TCAGAGTCCGAAGACAGACTTGAG-3' to generate a fragment of 1.2 kb. PCR conditions were 95°C 5 minutes, 30 cycles of 95°C 30 seconds for denaturation, 55°C 30 seconds for annealing and 72°C 1.5 minutes for extension, an extra extension at 72°C 15 minutes was included at the end of the procedure. Csrnp1 primers and RT-PCR was performed as previously described [12]. Real-time PCR was performed with the Mx3000P system (Stratagene) using SYBR Green. Samples contained 7.5 μL of Maxima SYBR Green qPCR Master Mix 2X with Rox (Fermentas), 0.5 μL of each primer (10 μM), 1 μL of template and 5.5 μL DEPC water. In negative controls cDNA was replaced by DEPC water. All PCR were done with 2 biological replicates. The Real-time conditions were 95°C for 10 min and 40 cycles of 95°C 30 seconds, 60°C 30 seconds and 72°C 30 seconds using the primers: β-actin F 5'-ATCTCTCATCAGGATGTCGAGGT-3', β-actinR 5'-AAGGCTTCCAGGTTTTCTCTCCG-3', csrnp1aF 5'-CTCTGAGTGAAGACAGACTTGAG-3' and csrnp1aR 5'-GTAACCAGGACAAACCGAAG-3'.

Whole Mount in situ Hybridization and Immunohistochemistry

ISH experiments were performed as described in Thisse et al. 2008 [30]. The RNA-probes were sensitized according to standard protocols. csrnp1a probe was synthesized using the AGENCOURT 22438433 EST (OpenBiosystems) as template. The following genes were kindly provided as cDNA clones and used as templates for making RNA probes: stall [31], gse [32], gata5 [33], tal1/scl [6], klf4 [11], spi1/pu.1 [6], gata1 [34], hba1 [35], hpf1 [36], amnc [37], vmsdc [37] and moya [38]. For immunohistochemistry, we used rabbit Anti-phospho-Histone H3 (H3-P, Upstate 07–424). Immunolabeling was carried out essentially as described in Sarrazin et al. 2006 [30]. To define the region for H3-P-positive cell counts, immunohistochemistry against H3-P was performed in embryos previously labeled by in situ hybridization with spi1/pu.1 or sel probes. H3-P signal was only counted inside the in situ area.

O-dianisidine and DAB Stains

O-dianisidine was performed as previously described [39]. The DAB stain solution was prepared as follow: 0.07 mM of O-dianisidine and DAB Stains at the concentration described by Robu et al. 2007 [18].
minutes in PBST, one more in PBS and finally incubated in the DAB stain solution. Embryos were observed after 3 hours of incubation.

Acridine Orange Staining
Embryos were stained according to Williams and Webb, 2000 [40]. Briefly, embryos were incubated for 20 min in 5 ug/ml acridine orange (Sigma) in E3 medium, washed five times for 5 min in E3 medium and observed under fluorescence microscopy.

Supporting Information
Figure S1 csrnp1a gene scheme, morpholino target sites, primers used and RT-PCR of csrnp1a. The complete csrnp1a genome sequence comprises 14,724 bp, which contains five exons (blue boxes) and 6 introns (red boxes). In the diagram morpholino hybridization sites are represented with black lines and in green lines represent the primers hybridization sites designed for RT-PCR. (B) RT-PCR of 24 hpf control embryos (C) and Splicing morphants (MoSpt) injected with 2,5 and 5 ng per embryo, showing the dose dependent decrease of csrnp1a mRNA. This decrease was reverted at 30 hpf, indicating that the sp morpholino is no longer efficient at this time.

Figure S2 Early mesoderm specification is not altered in csrnp1a knockdown condition. Expression of the mesodermal markers goosecoid (gsc) (A, D), notal (ntl) (B, E) and gata5 (C, F) detected by ISH at three different stages of gastrulation. No differences were detected between morphant and control embryos (100%, n = 37, 49 and 46 embryos respectively).

Figure S3 csrnp1a knockdown does not affect blood flow. Morphant embryos were incubated in the vasodilator SNAP, and its ability to rescue the reduction of blood cells was analyzed (A, B, C). csrnp1a knockdown phenotype was not rescue by SNAP treatment. (A) Control 100% n = 53; (B) Mo csnp1a atg 36,0% n = 21/57 of morphant phenotype; (C) Mocrsnp1a atg incubated with SNAP 33,9% n = 21/62 of morphant phenotype.

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In situ hybridizations against ventricular (vmhc) (D, E) and auricular (amhc) (F, G) markers show that injected embryos have normal heart development (100%; n = 43; n = 45 respectively). All are lateral views, anterior to the left. (H) Chart depicting the number of heartbeat in 10 seconds in control and csrnp1a morphant embryos.

Figure S4 csrnp1a morphants exhibit an increase in cell death due to hematopoietic toxicity which is not responsible for the reduction in blood cells in circulation. We analyzed cell death by acridine orange in embryos injected with control morpholin (A), csrnp1a morpholin (C), p53 morpholin (E), or a mixture of csrnp1a and p53 morpholinos (G). A clear increase in cell death is detected in csrnp1a morphant embryos (86,7%; n = 38/67), which is reversed by p53 co-injection (16,7%; n = 16/96). The cell blood phenotype was screened at 33 hpf using O-Dianisidine stain in (B) control, (D) csrnp1a, (F) p53 morphant embryos and in embryos co-injected with both morpholinos (H). The csrnp1a, p53 co-injected embryos (H; 39,6% n = 21/53) have the same penetrance of the blood phenotype as csrnp1a morphants (F; 35,7% n = 15/42) (red arrows). It worth mention that the slight head reduction exhibited by csrnp1a morphant embryos was also detected in co-injected embryos (black arrows). All are lateral views, anterior to the left.

Acknowledgments
We would like to thanks Dr. Miguel Allende for his support during the preparation of this manuscript. We thank Dr. Ariel Reyes for provide us the ntl, gsc, gata5 probes; Dr. Graham Lischke for tal1/scl and spl/pa1.1 probes; Dr. Tadashi Wada for klf1 probe; Dr. Ting Xi Liu for gata1 probe; Dr. Michael Green for hhb1 and Dr. Rachel Warga for lepl1 probe. Finally the authors are thankful to Emilio Vergara for expert fish care.

Author Contributions
Conceived and designed the experiments: JE CGF AG. Performed the experiments: JE CGF GS. Analyzed the data: JE CGF AG. Contributed reagents/materials/analysis tools: JE CGF AG. Wrote the paper: JE CGF AG.
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