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SAP30L (Sin3A-Associated Protein 30-Like) is Involved in Regulation of Cardiac Development and Hematopoiesis in Zebrafish Embryos

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ABSTRACT

The Sin3A-associated proteins SAP30 and SAP30L share 70% sequence identity and are part of the multiprotein Sin3A corepressor complex. They participate in gene repression events by linking members of the complex and stabilizing interactions among the protein members as well as between proteins and DNA. While most organisms have both SAP30 and SAP30L, the zebrafish is exceptional because it only has SAP30L. Here we demonstrate that SAP30L is expressed ubiquitously in embryonic and adult zebrafish tissues. Knockdown of SAP30L using morpholino-mediated technology resulted in a morphant phenotype manifesting as cardiac insufficiency and defective hemoglobinization of red blood cells. A microarray analysis of gene expression in SAP30L morphant embryos revealed changes in the expression of genes involved in regulation of transcription, TGF-beta signaling, Wnt-family transcription factors, and nuclear genes encoding mitochondrial proteins. The expression of the heart-specific nkx2.5 gene was markedly down-regulated in SAP30L morphants, and the cardiac phenotype could be partially rescued by nkx2.5 mRNA. In addition, changes were detected in the expression of genes known to be important in hemoglobin synthesis and erythropoiesis. Our results demonstrate that SAP30L regulates several transcriptional pathways in zebrafish embryos and is involved in the development of cardiac and hematopoietic systems. J. Cell. Biochem. 113: 3843–3852, 2012.

KEY WORDS: ZEBRAFISH; SAP30L; TRANSCRIPTION; MICROARRAY; HEMATOPOIESIS; CARDIOGENESIS

Acetylation and other covalent modifications of histones play fundamental roles in chromatin dynamics and regulation of gene expression. Deacetylation of histones is carried out by a multiprotein corepressor complex in which Sin3A is an essential scaffold protein. Sin3A is composed of domains that mediate protein–protein interactions and thereby forms a platform to which several enzymes, DNA-binding transcription factors, and other bridging proteins bind [Silverstein and Ekwall, 2005]. The core

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Sin3A-HDAC corepressor complex contains the histone deacetylases HDAC1 and HDAC2, the histone-binding proteins RbAp46 and RbAp48, the Sin3A-associated protein 18 (SAP18), SAP30, and SDS3 [Silverstein and Ekwall, 2005].

The Sin3A-associated proteins SAP30 and SAP30L (SAP30-like) have been shown to be present in various corepressor complexes [Laherty et al., 1998; Zhang et al., 1998; Viiri et al., 2006]. We have previously reported that these proteins bind DNA directly and this association is regulated by nuclear monophospholipids [Viiri et al., 2009b]. SAP30 and SAP30L are small and basic, and contain nuclear (NLS) and nucleolar (NoLS) localization signals [Viiri et al., 2006]. The main difference between the two proteins lies in their N-termini, where SAP30 contains a 38 amino acid insertion relative to SAP30L. Both proteins are able to localize to the nucleus or the nucleolus, and they can direct Sin3A to the nucleolus [Viiri et al., 2006].

While a range of biochemical and cell biological results implicate SAP30 and SAP30L in repression of gene expression, the function(s) of these proteins in the setting of a living organism has not been established. In order to study this, we chose the zebrafish as a model organism. The zebrafish has only one of these proteins, SAP30L [Viiri et al., 2009a], enabling us to study the role of SAP30L without interference of potentially overlapping functions of SAP30. Our results suggest an important role for SAP30L in diverse transcriptional pathways in zebrafish embryos.

MATERIALS AND METHODS

ZEBRAFISH MAINTENANCE AND MICROINJECTIONS

Wild type AB zebrafish were maintained under standard conditions at 28.5°C as described [Westerfield, 1995]. In microinjection experiments, embryos at the 1-4-cell stage were injected into the yolk sac with 1 nl of injection mix, which contained the morpholino, mRNA (where indicated) and rhodamine dextran. The PV830 Pneumatic PicoPump injection system (WPI, Inc.) was used, and the injection volume was calibrated to 1 nl using 1 mm Micrometer scale (S48, Ted Pella Inc.). The care of the animals was in accordance with the Finnish Laboratory Animal Welfare Act 62/2006 and the Laboratory Animal Welfare Ordinance 36/2006.

WHOLE-MOUNT IN SITU HYBRIDIZATION (WISH)

DIG-labeled sense and antisense probes for zebrafish SAP30L were prepared using the DIG RNA Labeling Mix (Roche Diagnostics) according to the manufacturer's instructions, as described [Thisse and Thisse, 2008]. Linearized plasmids containing the full-length SAP30L cDNA (in the sense or antisense orientation) under the control of the T7 promoter were used as templates. Zebrafish larvae were collected and processed as described [Thisse and Thisse, 2008] at 12, 24, 36, 48, and 72 hpf. Whole-mount in situ hybridization (WISH) using alkaline phosphatase detection with the BM Purple substrate (Roche Diagnostics) was carried out according to Thisse and Thisse [2008] with the following modifications: 2 ml tubes were used during the entire procedure, hybridization was carried out at +65°C, 130 ng of sense or antisense probe was used per reaction and a dilution ratio of 1:2,000 was used for the anti-DIG antibody (sheep anti-digoxigenin-AP Fab fragments, Roche Diagnostics). The stained fish were analyzed and photographed under a dissecting microscope. The localization of the staining was assessed by comparing to the anatomical reference images by Haffter et al. [1996].

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Expression of the SAP30L, nkx2.5 and ppox genes was assayed by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from zebrafish embryos and adult fish tissues with an RNeasy Mini Kit (Qiagen), and converted to cDNA using an iScript Select cDNA Synthesis Kit (Bio-Rad) and random primers according to the manufacturers' instructions. The housekeeping gene EF1A (Ensembl ID: ENSDARG00000020850) [Tang et al., 2007] was used as a normalization control. The primer sequences are described in the Supplementary data. qRT-PCR was performed by using an Evagreen Ssofast supermix kit (Bio-Rad) as instructed by the manufacturer. An average threshold cycle (Ct) value was calculated from two or three replicate samples. The mRNA levels (mean ± SD) are expressed relative to those of the housekeeping gene, EF1A. The PCR runs were repeated twice, and no significant differences between replicate runs were observed (data not shown). For the microarray experiments, the total RNA was extracted from whole embryos collected at 12 or 24 hpf.

ANTISENSE MORPHOLINO EXPERIMENTS

The translational start site of the zebrafish SAP30L mRNA (Ensembl ID: ENSDARG00000030213) was targeted with two independent antisense morpholinos: SAP30L-MO1 and SAP30L-MO2 (Fig. 2a, Supplementary Table I). Their sequences exhibited no significant similarity to other loci in a search of a zebrafish database. The random control morpholino (RC, see Supplementary Table I) has no gene targets or observable biological activity in the zebrafish. The p53-targeting morpholino was also used (Supplementary Table I). All morpholinos were purchased from Gene-Tools, LLC. The effective concentrations of morpholinos were experimentally titrated and are indicated in the figures or figure legends.

WESTERN BLOTTING

The efficacy of MO1 and MO2 in blocking the translation of the SAP30L mRNA was analyzed using a human anti-SAP30L antibody [Korkeamaki et al., 2008]. MO1-, MO2-, or RC-injected larvae were collected at 2 dpf (days post-fertilization) directly into 2× Laemmli buffer (10 larvae/50 μl), and lysed by boiling and passing through a pipette vigorously. The lysates were centrifuged for 5 min to remove the debris, and the soluble fraction was used for further analysis. The proteins were resolved on 12% SDS–PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences). The immunoblots were probed with an anti-SAP30L antibody, and the proteins were detected by enhanced chemiluminescence. In all zebrafish lysates, the anti-SAP30L-antibody recognized only a single band, the molecular weight of which is a few kilodaltons lower than in human control (lysed cells transfected with Myc-His-tagged SAP30L), as expected due to the presence of the tag and the larger size of human SAP30L.

ANALYSIS OF HEART MORPHOLOGY AND FUNCTION

Heart morphology at 5 dpf was assessed visually and categorized as normal or deformed, examples of which are shown in Supplementary Figure S3. Heart rates were determined visually in unanaesthetized morphant and control larvae at 5 dpf. Ventricular and atrial performance was analyzed on 10–20 s time-lapse videos. The longitudinal fractional shortening was calculated according to the formula $(LDd - LDs)/LDd$, where *LDd* is the longitudinal diameter at diastole and *LDs* the diameter at systole.

HISTOLOGICAL STAINING

The morphant and control larvae were fixed in a 4% formaldehyde–PBS solution at +4°C and embedded in 2% agarose. The samples were dehydrated by incubating them in a series of alcohol solutions (70%, 96%, and absolute ethanol) for 1–2 h in each solution, and finally in xylene for at least 1 h. The samples were then embedded in paraffin and 5 μm-thick sections were cut in longitudinal orientation. The sections were fixed on glass slides and deparaffinised by incubating them twice in xylene (á 4 min), twice in absolute ethanol (á 3 min), twice in 96% ethanol (á 3 min), once in 70% ethanol, and rinsing once with water. Hematoxylin–eosin staining was performed by incubating the samples in Mayer's hematoxylin (2 min), running water (2 min), water (1.5 min), 70% ethanol (15 s), eosin Y (15 s), 96% ethanol (30 s, twice), absolute ethanol (1 min, twice), and xylene (1–4 min). After this the slides were mounted and pictures were taken under a BX60 microscope using the CellˆD program (Olympus).

O-DIANISIDINE STAINING

Detection of hemoglobin by *o*-dianisidine staining was performed as described previously [Ransom et al., 1996]. Larvae stained at 3 dpf were scored as normal, reduced, or severely reduced based on their qualitative level of staining which was assessed visually. In the normal category, staining was judged to be equivalent to that of wild type larvae, reduced staining was visibly decreased as compared to wild type, and in the severely reduced category, the larvae showed little or no staining.

mRNA RESCUE

The mRNA rescue experiments were performed by injecting simultaneously SAP30L-MO1 or SAP30L-MO2 and a capped synthetic mRNA encoding the gene of interest. We used various doses of mRNA in the injections, and the data were pooled for analysis. The mRNA was synthesized using a T7 mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions. Briefly, a plasmid template linearized with *Hind*III was mixed thoroughly with components of the kit and incubated at +37°C for 2 h, after which the template DNA was removed by a DNase treatment. The synthesized mRNA was purified using a MEGAclear kit (Ambion) according to the manufacturer's protocol and further concentrated by ammonium acetate precipitation. The templates used in the synthesis reactions had been created by cloning the desired cDNA into the pcDNA3.1/*myc*His(−)A-vector (Invitrogen) using *Eco*RI and *Hind*III restriction sites. The constructs contained full-length zebrafish SAP30L (drSAP30L) or full-length zebrafish nkx2.5 (Nkx2.5), each without a stop codon.

STATISTICAL ANALYSES

Quantitative data are given as mean ± standard deviation. When appropriate, differences between groups were compared by using the two-tailed Student *t*-test or the χ^2 test for cross tabulations. A *P*-value of less than 0.05 was considered statistically significant. Statistical calculations were performed using PASW Statistics version 18.

MICROARRAYS

200 ng of total RNA was amplified and labeled with Cy3 using a Low Input Quick Amp Labeling kit (Agilent Technologies). The samples were processed using an RNA Spike In kit (Agilent Technologies). 1.65 μg of each Cy3-labeled sample was hybridized to Agilent's 4 × 44K Zebrafish V3 Gene Expression Microarray overnight at 65°C in the buffers of Agilent's Gene Expression Hybridization kit. The arrays were washed according to the manufacturer's instructions and scanned with an Agilent Technologies' scanner (model G2565CA), using scan profile AgilentHD_GX_1Color. Numerical data were extracted with Agilent's Feature Extraction software, version 10.7.1., using grid 026437_D_F_20100719 and protocol GE1_107_Sep09.

MICROARRAY DATA ANALYSIS

The R programming language and environment [R Development Core Team, 2008] and its Bioconductor module [Gentleman et al., 2004] were used for analysis of data from the microarrays. The raw data were normalized with quantile normalization, and the quality of the data was checked with several quality control methods. Statistical testing was performed with the Limma package [Smyth, 2005], and stringent filtering thresholds were used for both time point comparisons (fold change FC$\geq$|3| and *P*-value $P \leq 0.001$).

RESULTS AND DISCUSSION

SAP30L mRNA IS EXPRESSED DURING EMBRYOGENESIS AND IN ADULT ZEBRAFISH TISSUES

SAP30 and SAP30L have been implicated in the regulation of gene repression through the Sin3A-corepressor complex [Laherty et al., 1998; Zhang et al., 1998; Viiri et al., 2006, 2009b]. Their function in vivo remains unknown and we therefore set out to examine the function of SAP30L using the zebrafish as a model. Contrary to the human and mouse genomes, only one member of the SAP30 family has thus far been identified in the zebrafish genome, and its derived protein sequence more closely resembles human SAP30L than SAP30 (Supplementary Fig. S1; Viiri et al., 2009a).

We studied the expression of the SAP30L gene during zebrafish embryogenesis in whole embryos by qRT-PCR, and determined the localization of the expression by WISH at 12, 24, 36, 48, and 72 h post-fertilization (hpf) (Fig. 1a and b). The results show that SAP30L mRNA is widely expressed during embryogenesis (Fig. 1a and b). The expression is strongest in the brain, including forebrain, midbrain, and hindbrain, at the 24, 36, 48, and 72 hpf time points (Fig. 1a). The pectoral fin buds (at 36 and 48 hpf) and pectoral fins (72 hpf) also show strong expression of the mRNA. Next to the pectoral fin buds, staining is seen also in the region of common cardinal vein (see dorsal view in Fig. 1a) starting at 36 hpf. Furthermore, the heart area

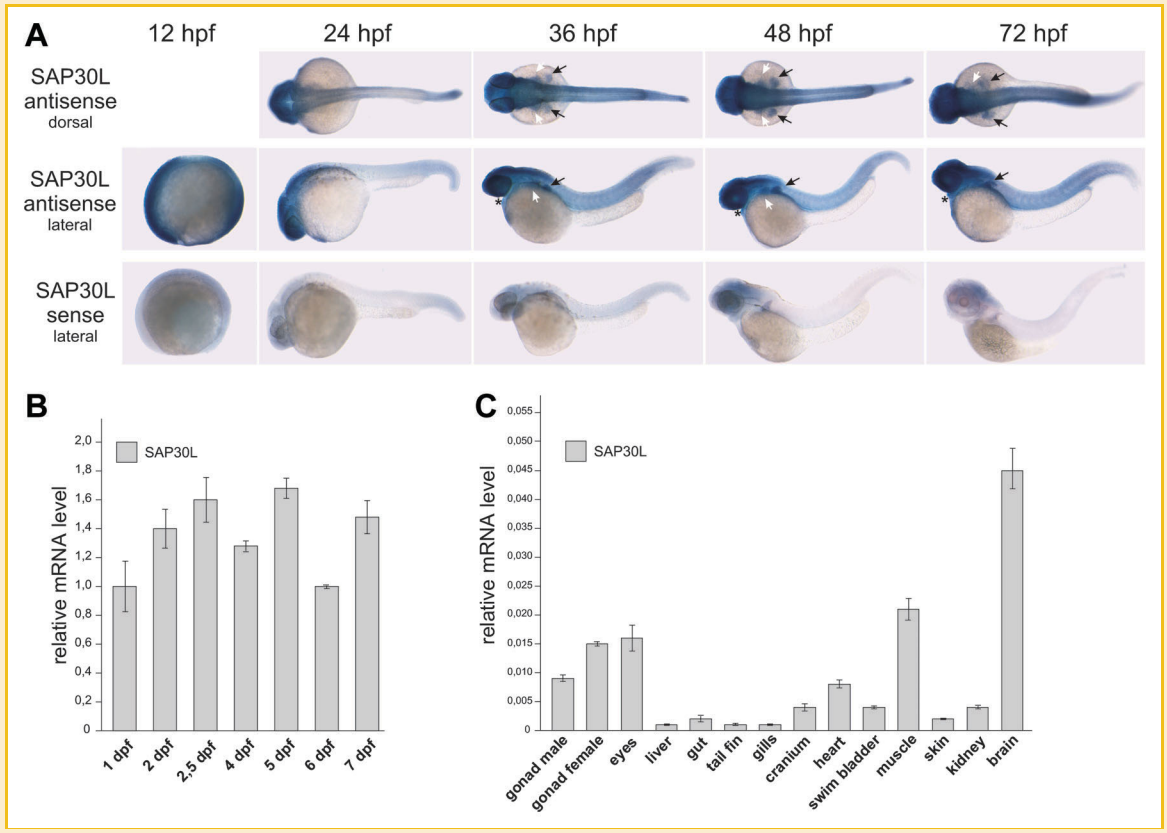

Fig. 1. Expression of SAP30L mRNA in zebrafish embryos, larvae, and adult tissues. A: WISH analysis of SAP30L mRNA expression during embryogenesis at indicated time points. Staining with an antisense probe is shown in dorsal (top row) and lateral (middle row) views, and a control sense-strand probe is shown in lateral view (bottom row). The black asterisk points out the heart next to it, the black arrow indicates the pectoral fin bud/pectoral fin, and the white arrow indicates the region of the common cardinal vein. B: qRT-PCR analysis of SAP30L mRNA in whole embryos during embryogenesis. SAP30L mRNA levels are normalized to the level at 1 day post-fertilization (dpf). C: qRT-PCR analysis of SAP30L mRNA in adult tissues. SAP30L mRNA levels (mean ± SD) are expressed relative to those of the housekeeping gene, EF1A. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

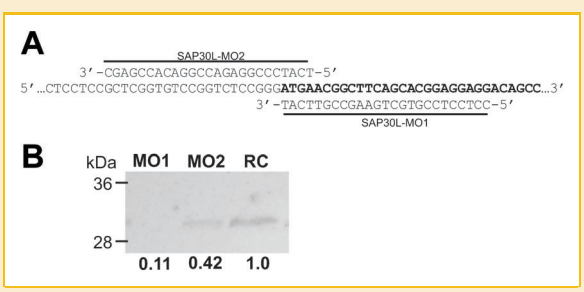

Fig. 2. The morpholinos used to target the SAP30L mRNA. A: Sequence of the coding strand of the zebrafish SAP30L gene, showing the 5′-untranslated region (normal font) and part of the first exon (boldface starting from the ATG-codon). Complementarity of the sequences of the morpholinos SAP30L-MO1 and SAP30L-MO2 to the coding strand of the gene is illustrated. B: Western blotting analysis of SAP30L protein levels in 2 dpf larvae injected with SAP30L-MO1 (MO1), SAP30L-MO2 (MO2), or random control morpholino (RC). Morpholino concentrations used: 400 μM for MO1, 600 μM for MO2, 600 μM for RC. The numbers under the lanes indicate band intensity relative to RC. The lysates contain identical numbers of larvae per volume of lysis buffer, and equal volume of sample was loaded on the gel in each case.

shows positive staining, first seen in the pericardial cavity (24 hpf) and later in the heart itself. In addition, moderate expression of SAP30L mRNA is seen in the trunk (including the area of the intermediate cell mass, ICM), and some embryos show weak staining in the blood island at 24 hpf. In adult fish, strong expression is seen in the brain and moderate expression in muscle, eye, gonad, and heart tissues (Fig. 1c).

DESIGN AND VALIDATION OF THE SAP30L-TARGETING MORPHOLINOS

In zebrafish embryos, morpholino oligonucleotides (MO) can be used to knock down expression of genes of interest. We designed two translation-blocking morpholinos (MO1 and MO2) which target the translation initiation site in the SAP30L mRNA (Fig. 2a). In an in vitro translation assay, a specific and dose-dependent inhibition of translation of SAP30L mRNA was detected with the MO1 but not with RC control (Supplementary Fig. S2). The ability of morpholinos to block translation of SAP30L was further investigated by using the SAP30L antibody and Western blotting. A significant reduction in SAP30L protein levels was detected in MO1- and MO2-treated zebrafish embryos at 2 dpf, demonstrating the efficacy of these

morpholinos in down-regulating the expression of the SAP30L protein in vivo (Fig. 2b).

KNOCKDOWN OF SAP30L EVOKES CARDIAC DEFECTS AND REDUCED HEMOGLOBIN LEVELS

In order to investigate the role of SAP30L in zebrafish development, we injected morpholinos (MO1 or MO2) into 1- to 4-cell stage embryos, and observed their development for 5–6 dpf. The SAP30L morphants showed prominent and progressive pericardial edema. Importantly, most of the SAP30L morphants exhibited deformed cardiac morphology (85–89%; Fig. 3a, Supplementary Fig. S3), with the hearts remaining string-like and visible on a single plane, in contrast to the three-dimensional complexity of the healthy hearts in the control larvae (Fig. 3b). Cardiac function was assessed by determining the heart rates and the contractility of the chambers. The results demonstrate significantly reduced heart rates in MO1- and MO2-injected morphants (Fig. 3c), with the differences detectable already at 3 dpf. Similarly, a substantial decrease in the longitudinal fractional shortening values was seen in both the atrium and the ventricle, as compared to control hearts (Fig. 3d). Videos illustrating the diminished cardiac function can be found in the Supplementary data (SV1-3). The results are compatible with the WISH and qRT-PCR analyses which showed expression of SAP30L in the developing and adult heart (Fig. 1a and c), and suggest that the phenotype results specifically from the knockdown of SAP30L.

In some SAP30L morphants, the red blood cells seemed paler as compared to those in control larvae. In a Sin3B-knockout mouse, impaired erythroid differentiation has been reported, with a 43% reduction in the number of circulating red blood cells and a 37% reduction in hemoglobin levels [David et al., 2008]. In order to determine hemoglobin levels in the SAP30L morphants, *o*-dianisidine staining was performed on 3 dpf embryos (see examples in Supplementary Fig. S4). As summarized in Figure 3e, the morphants exhibited reduced staining, indicating reduced hemoglobin levels in the red blood cells. The observed effect on erythroid differentiation is consistent with the fact that SAP30L expression is seen at the area of the common cardinal vein and ICM,

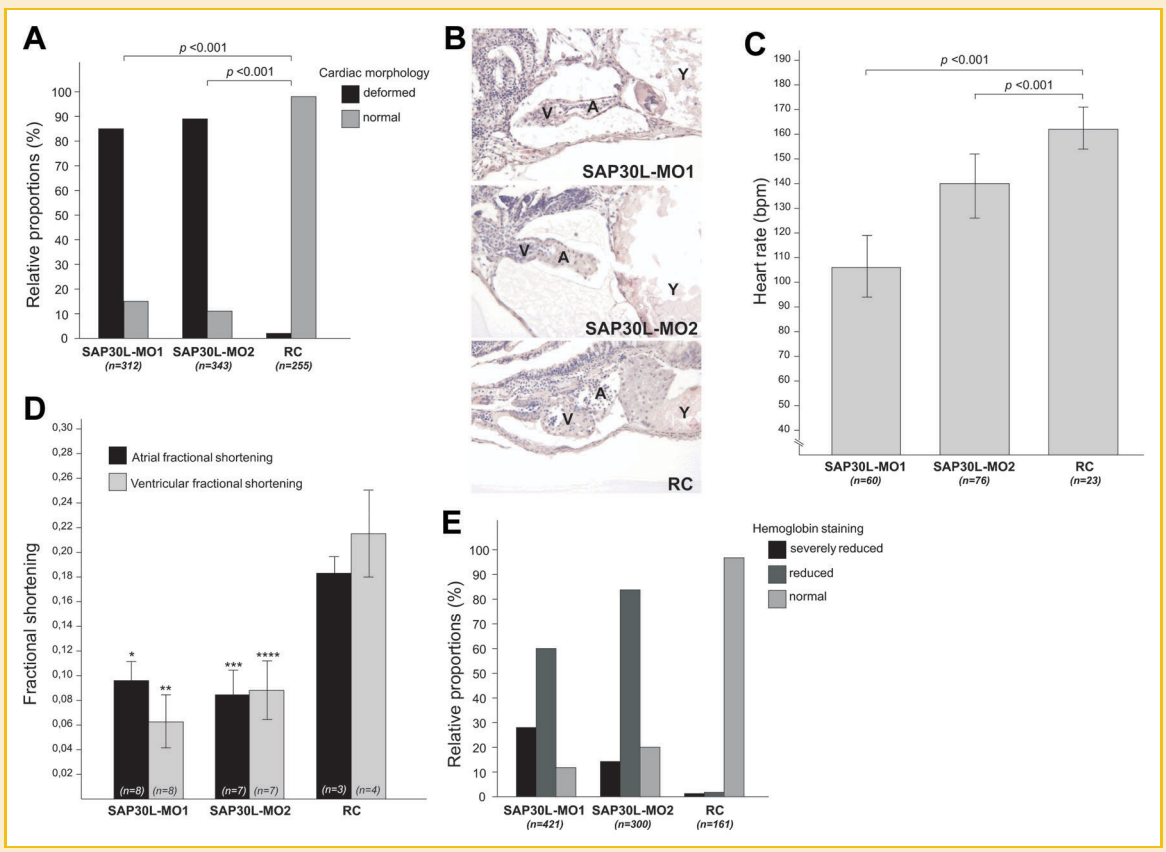

Fig. 3. The phenotype induced by knockdown of SAP30L involves defects in the heart and in hemoglobinization of red blood cells. A: The majority of the SAP30L morphant larvae have deformed hearts (see close-up images of normal and deformed hearts in Supplementary Fig. S2). B: Histological analysis of cardiac morphology. Hematoxylin–eosin staining of sections reveals string-like appearance of the heart in the MO1- and MO2-injected larvae, in contrast to the RC-injected controls. V, ventricle; A, atrium; Y, yolk sac. C: SAP30L morphant larvae exhibit reduced heart rates. Heart rates (beats per minute, bpm) are represented as mean bpm ± SD. D: Both atrial and ventricular performance in SAP30L morphant hearts are impaired, as shown by the decreased longitudinal fractional shortening values (represented as mean ± SD). $^{*}P = 0.002$; $^{**}P < 0.001$; $^{***}P = 0.005$; $^{****}P = 0.006$, as compared to RC control. The morpholino concentrations used in A–D: 300–750 μM for MO1, 500–600 μM for MO2, 400–750 μM for RC. E: SAP30L morphants exhibit defects in hemoglobinization of red blood cells (see examples in Supplementary Fig. S4). MO1-, MO2-, or RC-injected larvae are scored as normal, reduced, or severely reduced based on hemoglobin staining by *o*-dianisidine. Morpholino-concentrations used: 400 μM for MO1, 600 μM for MO2, 600 μM for RC. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

where developing erythrocytes are present [Davidson and Zon, 2004].

In addition, some morphant larvae were slightly smaller than control larvae, and showed edema in the head and brain (data not shown).

The phenotypes obtained with both SAP30L-targeting morpholinos were essentially similar, with MO2-morphants showing slightly delayed onset, which concurs with the less decreased level of SAP30L protein in these morphants as compared to MO1-morphants (Fig. 2b). Since morpholinos are known to induce off-target p53-mediated effects [Robu et al., 2007], we co-injected both morpholinos with the p53-morpholino. Larvae injected with SAP30L-MO2 alone or together with the p53-morpholino, did not differ in phenotype. SAP30L-MO1 morphants exhibited a slightly alleviated but essentially similar phenotype when co-injected with the p53-morpholino (data not shown).

One way to demonstrate the specificity of the observed phenotype is to rescue it by co-injecting the morpholino with the target mRNA [Eisen and Smith, 2008]. In our case, this strategy could be exploited only with MO2, since it targets the SAP30L mRNA upstream of the start codon. No rescue of the reduced hemoglobin staining but a tendency towards a less severe cardiac phenotype could be observed in the rescued morphant larvae (data not shown). The incomplete rescue may be due to various reasons, such as the short half-life of the injected mRNA, strict temporal or spatial constraints on the expression at certain developmental stages, inability of the mRNA to reach the right tissue, or the fine balance between under- and over-expression of the protein in the embryos and larvae.

SAP30L IS INVOLVED IN MULTIPLE TRANSCRIPTIONAL PATHWAYS DURING ZEBRAFISH EMBRYOGENESIS

SAP30L is a part of the Sin3A-HDAC multiprotein corepressor complex [Viiri et al., 2006]. In order to elucidate the transcriptional pathways in which SAP30L participates during zebrafish embryogenesis, we performed a microarray analysis of embryos in which expression of SAP30L was knocked down. To avoid any secondary effects on gene expression, we chose early time points at 12 and 24 hpf. It has been reported previously that most developmentally regulated genes reach their peak expression by 24 hpf, with a sharp increase in transcript accumulation at 12 hpf [Mathavan et al., 2005]. In our analysis, 205 genes were up-regulated and 174 genes down-regulated at the 12 hpf time point, whereas at 24 hpf, the respective numbers were 143 and 64 (Fig. 4, Supplementary Fig. S5). Of the affected genes, 46 were identified at both time points and showed similar alterations in expression. Since SAP30L is part of a transcriptional repressor complex, its knockdown may be expected to lead to more up-regulation than down-regulation of genes. Our results bear out this expectation and are in line with the published microarray data for Sin3, another member of the same repressor complex. RNAi-mediated knockdown of *Drosophila* SIN3 resulted in induction of 364 genes but only 35 genes were repressed [Pile et al., 2003]. Similarly, in mouse cells in which Sin3A had been knocked out, the majority of the differentially expressed genes were up-regulated [Dannenberg et al., 2005]. The results of knockdown of *Drosophila* SIN and the mouse mSin3A were similar in that mainly genes involved in cytosolic and mitochondrial energy-generating pathways were affected. Our data on knockdown of SAP30L in *Danio rerio* show no marked changes in expression of the genes reported in the *Drosophila* SIN and mouse Sin3A data sets. Instead, expression of several nuclear genes encoding mitochondrial proteins was affected by knockdown of SAP30L. These include timm8a, cyb5b, ppox, alas2 and fars2 (Fig. 4, Supplementary Fig. S5).

SAP30L was originally identified in our laboratory as a TGF-beta-inducible transcript in a cell culture system that models differentiation of the intestinal epithelium [Lindfors et al., 2003]. Interestingly, down-regulation of SAP30L in the zebrafish resulted in strong induction of Smad2, a mediator in the TGF-beta signaling pathway. The 150 kDa TGF-beta-1-binding protein was similarly up-regulated at 12 hpf, whereas the bone morphogenetic factor 5 (BMP5), a member of the TGF-beta superfamily, was slightly down-regulated. These results taken together with our previous findings suggest a role for SAP30L in TGF-beta signaling events.

In gene ontology analysis, the pathway with the most hits is associated with regulation of transcription. The same result was obtained when the analysis was performed on the genes that showed the largest differences in their expression. Indeed, several transcription factors show altered expression in our microarray analysis (Fig. 4, Supplementary Fig. S5). Among them are the Wnt-family transcription factor Wnt7b, which is involved in the development of vasculature, lung, and kidney [Shu et al., 2002; Lobov et al., 2005; Rajagopal et al., 2008; Yu et al., 2009; Lin et al., 2010], and Wnt2, which has been shown to be associated with cardiogenesis and several other developmental processes [Alexandrovich et al., 2006; Wang et al., 2007; Goss et al., 2009, 2011; Sousa et al., 2010]. In addition, Wisp3, a protein involved in modulation of Wnt and BMP signaling [Nakamura et al., 2007], was affected.

NKX2.5 EXPRESSION IS REDUCED IN SAP30L KNOCKDOWN EMBRYOS

One of the genes down-regulated at 12 hpf in the microarray analysis of SAP30L morphants, Nkx2.5 (Fig. 4), is a well-known regulator of heart development [Chen and Fishman, 1996; Targoff et al., 2008; Reamon-Buettner and Borlak, 2010]. The down-regulation of the Nkx2.5 gene was confirmed by qRT-PCR, which showed a threefold decrease in its expression in SAP30L-MO1-treated embryos, and a sixfold decrease in SAP30L-MO2-treated embryos (Fig. 5a). Interestingly, co-injection of Nkx2.5 mRNA with SAP30L-MO1 could partially rescue the deformed cardiac morphology in the SAP30L morphants (Fig. 5b). Previous studies in other model organisms have implicated Nkx2.5 in various cardiac developmental processes, including progenitor specification and proliferation, heart tube extension and looping, and chamber morphogenesis [Targoff et al., 2008]. Our results suggest that transcription of Nkx2.5 is regulated by a SAP30L-containing repressor complex in zebrafish embryos. As SAP30L is a member of a large corepressor complex and binds DNA only nonspecifically, its observed effect on expression of Nkx2.5 is likely to be indirect. Interestingly, some members of the Sin3A corepressor complex are known to regulate expression of genes critical for cardiac morphogenesis and function in both the zebrafish and mice. These include HDAC1 [Pillai et al., 2004; Montgomery et al., 2007], HDAC2

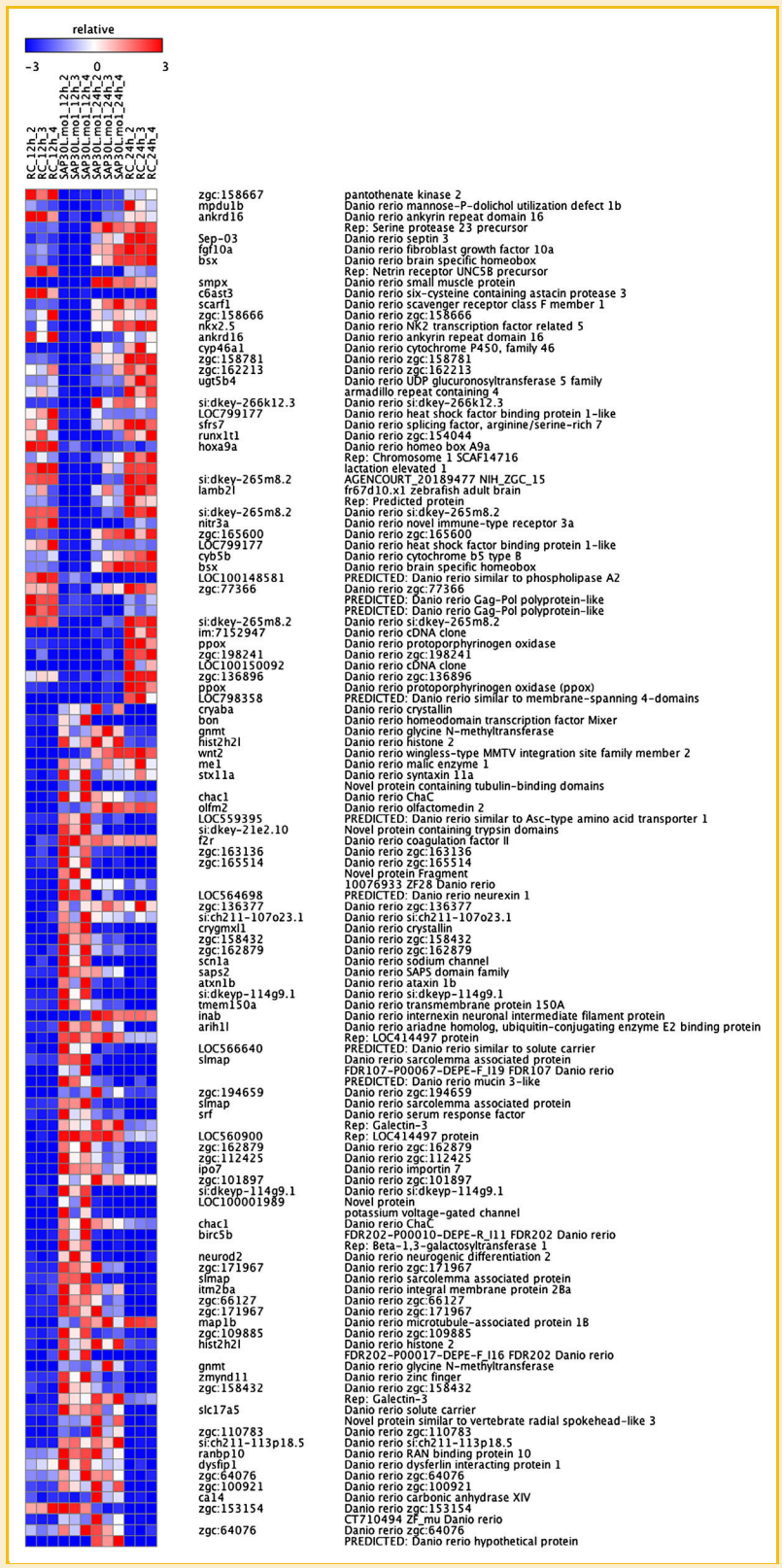

Fig. 4. Microarray analyses of zebrafish embryos (at 12 and 24 hpf) treated with SAP30L-MO1 or random control morpholino (RC). The 104 genes showing the largest differences in expression, ranked according to both the fold-change value and the *P*-value are shown for both time points and in all three parallel samples (2, 3, 4). Blue color denotes down-regulation, red up-regulation, and white no change. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

[Montgomery et al., 2007], HDAC3 [Farooq et al., 2008], and mSDS [Amsterdam et al., 2004].

GENES INVOLVED HEMOGLOBIN SYNTHESIS ARE DOWN-REGULATED BY SAP30L KNOCKDOWN

Consistent with the finding of diminished hemoglobin staining in the morphant larvae, we observed a marked reduction in expression of genes involved in hemoglobin synthesis and erythropoiesis (ppox, alas2, and ba1l globin-like gene) at 24 hpf (Fig. 4, Supplementary Fig. S5). The zebrafish ppox, a protoporphyrinogen oxidase, is expressed in the ICM as early as 22 hpf, similar to other genes involved in hematopoiesis, including alas2, gata1, and β-spectrin [Detrich et al., 1995; Brownlie et al., 1998; Liao et al., 2000]. Down-regulation of ppox was confirmed by qRT-PCR, which showed a sixfold decrease in its expression in SAP30L-MO1-treated embryos and a 4.8-fold decrease in SAP30L-MO2-treated embryos (Fig. 5c). In the future, it remains to be elucidated whether the lower hemoglobin levels observed after knockdown of SAP30L are due to impaired erythropoiesis, a block in heme biosynthesis, or some other mechanism. Our results suggest that one function of SAP30L may be to silence the expression of a factor that inhibits heme synthesis or erythropoiesis.

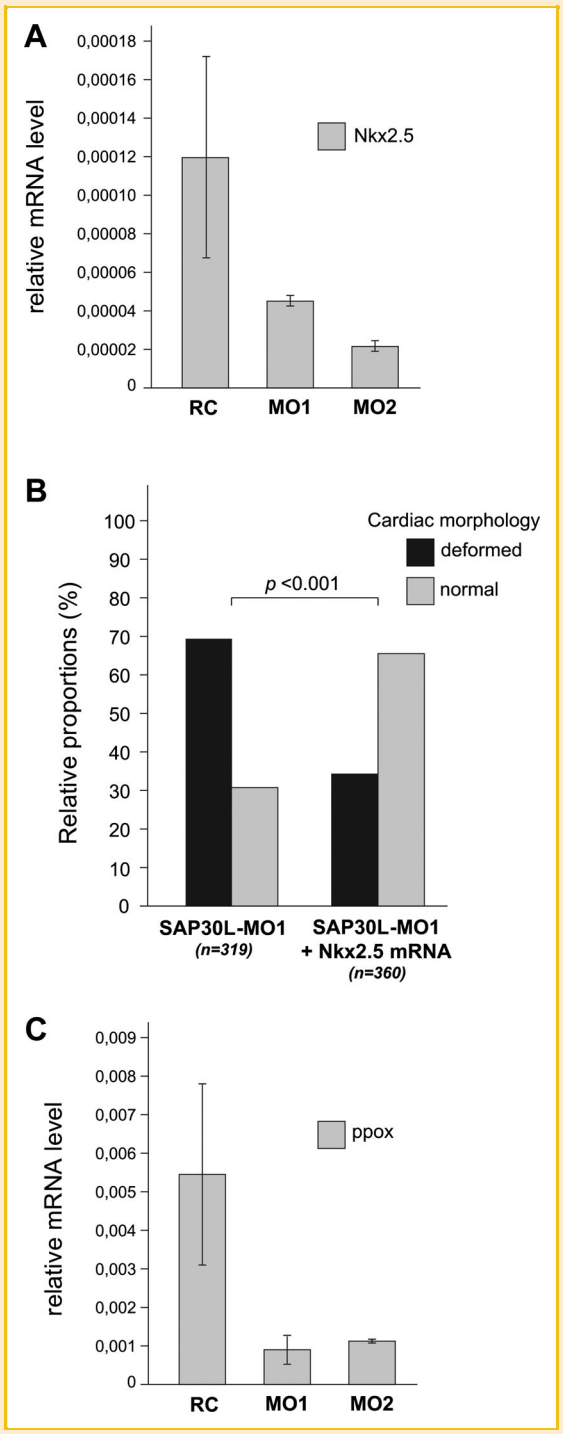

Fig. 5. Expression of Nkx2.5 and ppox mRNAs is down-regulated during SAP30L knockdown. A: Nkx2.5 mRNA expression is reduced in both SAP30L-MO1- and SAP30L-MO2-treated embryos at 12 hpf relative to RC-treated embryos. Nkx2.5 mRNA levels (mean ± SD) are expressed relative to those of the housekeeping gene, EF1A. B: The number of larvae exhibiting cardiac deformation is significantly reduced when treated with both SAP30L-MO1 (400 μM) and Nkx2.5 mRNA (20–50 pg per embryo), as compared to larvae treated with SAP30L-MO1 only. C: ppox mRNA expression is reduced in SAP30L-MO1- and SAP30L-MO2-treated embryos at 24 hpf compared to RC-treated embryos. ppox mRNA levels (mean ± SD) are expressed relative to those of the housekeeping gene, EF1A. The morpholino concentrations used in A and C: 400 μM for MO1, 600 μM for MO2, and 600 μM for RC.

CONCLUSION

SAP30 and SAP30L, members of the well-known Sin3A corepressor complex, have been previously implicated in repression of gene expression. However, the function(s) of these proteins in the setting of a living organism has not been established. Zebrafish was chosen as a model organism because it has only one of these proteins, SAP30L, enabling us to study its role without interference of potentially overlapping functions. Here we have demonstrated that SAP30L is ubiquitously expressed in the zebrafish and participates in the regulation of transcription of multiple genes. Morpholino-mediated knockdown of SAP30L protein results in disruption of cardiac development and function, and a reduction in hemoglobinization of red blood cells. Our results give important in vivo evidence of the role and function of SAP30L during the zebrafish embryogenesis.

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