Ssrp1a controls organogenesis by promoting cell cycle progression and RNA synthesis

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SUMMARY

Tightly controlled DNA replication and RNA transcription are essential for differentiation and tissue growth in multicellular organisms. Histone chaperones, including the FACT (facilitates chromatin transcription) complex, are central for these processes and act by mediating DNA access through nucleosome reorganisation. However, their roles in vertebrate organogenesis are poorly understood. Here, we report the identification of zebrafish mutants for the gene encoding Structure specific recognition protein 1a (Ssrp1a), which, together with Spt16, forms the FACT heterodimer. Focussing on the liver and eye, we show that zygotic Ssrp1a is essential for proliferation and differentiation during organogenesis. Specifically, gene expression indicative of progressive organ differentiation is disrupted and RNA transcription is globally reduced. Ssrp1a-deficient embryos exhibit DNA synthesis defects and prolonged S phase, uncovering a role distinct from that of Spt16, which promotes G1 phase progression. Gene deletion/replacement experiments in Drosophila show that Ssrp1b, Ssrp1a and N-terminal Ssrp1a, equivalent to the yeast homologue Pob3, can substitute Drosophila Ssrp function. These data suggest that (1) Ssrp1b does not compensate for Ssrp1a loss in the zebrafish embryo, probably owing to insufficient expression levels, and (2) despite fundamental structural differences, the mechanisms mediating DNA accessibility by FACT are conserved between yeast and metazoans. We propose that the essential functions of Ssrp1a in DNA replication and gene transcription, together with its dynamic spatiotemporal expression, ensure organ-specific differentiation and proportional growth, which are crucial for the forming embryo.

KEY WORDS: Ssrp1, Cell cycle, Hdac, Liver, Organogenesis, Zebrafish, Drosophila

INTRODUCTION

Expansion of progenitor pools and their tissue-specific differentiation are central for the formation of functional organs. Complex signalling cascades controlling organ growth and differentiation have been identified, but the associated basic cellular processes, such as regulation of DNA accessibility, in the developing embryo are still poorly understood. DNA accessibility, allowing the passage of RNA polymerase II and replication forks, is pivotal for coordinated gene expression and cell proliferation. Histone chaperones mediate DNA accessibility by structural destabilisation of nucleosomes and deconvolution of higher-order chromatin structure (Ransom et al., 2010). One histone chaperone, the FACT (facilitates chromatin transcription) complex consists of Spt16 (suppressor of Ty16; also known as Supt16h in zebrafish) and Ssrp1 (Structure specific recognition protein 1), which are both essential for nucleosome reorganisation and conserved across metazoans and plants (Formosa, 2005) (for genotyping details, see Zebrafish International Resource Center). The following strains were bred under standard conditions (Westerfield, 2000): Tg(XIEef1a1:GFP)s854 (Field et al., 2003), clampedΔ189, clampepg102, cecylil: Tg(Efα:mK02-zCdt1(1/90))e001b, Tg(Efα:mAG-zGem/(1/100))e001b (Sugiyama et al., 2009) and tps5Δ17 (Berghmans et al., 2005) (for genotyping details, see Zebrafish International Resource Center).

Positional cloning of clampedΔ189

Meiotic mapping followed standard protocols (Geisler, 2002). Sibling and cmpΔ189 mutant CDNAS were sequenced for ssrp1a, rad9a, zgc:113229, med19a and slc43a1a. For genotyping of homozygous cmpΔ189 embryos, dCaps Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) was used to design primers creating a Hinfl restriction site including the ssrp1aΔ189 mutation. The following primers were used for PCR amplification from genomic DNA: forward 5'-AGCCTATAGCAACAGATGG-3' and reverse 5'-CAGTTCCTCAATCCACCTGTCAT-3'. PCR amplification and subsequent Hinfl digest from homozygous mutant, wild-type and heterozygous embryos confirmed the identified lesion (supplementary material Fig. S1). ‘MO-ssrp1a’ (MO-ATG-ssrp1a, 5'-CGTAAACT-CGAGTGTTCTCCAT-3'; Gene Tools) and standard ‘MO-control’ were injected into one-cell-stage wild-type or Tg(XIEef1a1:GFP)s854 zebrafish.
Zebrafish immunostaining and in situ hybridisation

Labelling was performed as described (Ober et al., 2006). The following antibodies were used: rabbit anti-Prox1 (1:1000; Chemicon), mouse anti-2F11 (1:1000; gift from J. Lewis, Cancer Research UK, London, UK), mouse anti-BrU [1:20; Developmental Studies Hybridoma Bank (DSHB)] and rabbit anti-cleaved Caspase 3 (1:75; Cell Signaling Technology). Fluorescently conjugated secondary antibodies were obtained from Jackson Laboratories. Staining was visualised with a Zeiss LSM5 Pascal Exciter confocal microscope. Images were processed and cell numbers determined with Velocity image analysis software (Improvision).

The following probes were used for in situ hybridisation: ahi5 (also known as aito7) (Masai et al., 2000), ceruloplasmin (Korzh et al., 2001), cce (Shkumatava et al., 2004), foxal (Odenthal and Nüsslein-Volhard, 1998), group specific component (Noël et al., 2010), mdm2 (Chen et al., 2005), p21 (cyclin-dependent kinase inhibitor 1a (cdk1a1)) (Chen et al., 2005), pcnu (Leung et al., 2005) and ptf1 (Lin et al., 2004).

BrdU incorporation assay

Embryos were incubated for 30 minutes at 28.5°C in 10 mM 5-bromo- deoxyuridine (BrdU) with 15% DMSO in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) and prior to immunolabelling were treated with 2 M HCl for one hour at room temperature.

Global RNA labelling

Embryos were incubated in 10 mM ethynyluridine (EU; Invitrogen) with 15% DMSO in embryo medium for one hour at 28.5°C. Click-it detection was performed following manufacturer’s instructions (Invitrogen).

Mitotic index analysis

Foregut-containing tissue was isolated manually in Hank’s balanced salt solution with 5% fetal calf serum on ice and passaged through a 40 μm filter. Single-cell suspensions were stained with Vybrant DyeCycle Ruby (Invitrogen) and analysed by fluorescence-activated cells sorting (FACS) (Calibur, Becton Dickinson). Raw data were analysed by FlowJo software.

Drosophila molecular biology, genetics and histology

Zebrafish genes were cloned into a modified pKC26 UAS vector containing an attB site and a 3’ V5 epitope-tag sequence. Plasmids were inserted into the attP site-containing locus VIE-260b to generate transgenic lines (for details, see Hadjiconomou et al., 2011). Genetic crosses are summarised in supplementary material Table S2. Dissected tissues were processed as described (Hadjiconomou et al., 2011) and labelled using mouse mAb24B10 (1:75; DSHB), rat anti-Elav (1:50; DSHB), rabbit anti-PH3 (1:100; Millipore/Upstate), rabbit anti-αPKC (1:100; sc-216, Santa Cruz) and mouse anti-α-TUB (1:500; Invitrogen) and fluorescein-labelled F(ab)’2 fragments from Jackson ImmunoResearch Laboratories as secondary antibodies. Images were collected with a Bio-Rad/Zeiss Radiance2100 or a Leica TCS SP5 II confocal microscope. Samples for scanning electron microscopy were prepared using standard procedures (Cheyette et al., 1994) and imaged with a JEOL 35CF microscope.

qPCR

Quantitative PCR (qPCR) was performed using primer sets for ssrp1a and ssrp1b (ssrp1aF 5’-CTTACCACCTCTGTCTCTACAA-3’, ssrp1bF 5’-TGAGAGGGATTAAGTGATAGACG-3’, ssrp1aR 5’-TCTCCACCTCTTCTGCTCAT-3’; ssrp1bR 5’-TCCCGCCTTCTCTCCATGAGATG3’, rpl13F 5’-TCTGAGAGGGATTAAGTGATAGACG-3’, rpl13R 5’-TCGAGAGGACTGTAAGAGGTATGC-3’). mRNA was extracted from five to ten embryos and processed. qPCR (60°C annealing temperature) was carried out using ABSolute SYBR Green Mix (Thermo) on the ABI Prism 7000 RTPCR Detection system. Expression values were normalized using rpl13 and compared using Student’s t-test. Unless otherwise indicated, three independent experiments were assessed with three samples each.

RESULTS AND DISCUSSION

The clamped819 mutation disrupts organ growth and differentiation

The clamped819 (cmp819) mutant was previously identified in a forward genetic screen for factors essential for endodermal organogenesis (Ober et al., 2006). cmp819 embryos display liver and pancreas hypoplasia at 48 hours post-fertilisation (hpf, Fig. 1A-D), and smaller fins, head and eyes from 40 hpf (Fig. 2B-E). Liver progenitors arise from the foregut endoderm and aggregate into the liver bud (Field et al., 2003). Concomitant with liver-bud outgrowth, hepatoblasts begin to differentiate into functional hepatocytes and biliary epithelial cells. From about 50 hpf, the liver grows rapidly. Quantification of hepatic progenitors in cmp819 mutants using Prox1 expression showed a 40% reduction at 48 hpf (Fig. 1B-D). ceruloplasmin (cp), which encodes a plasma protein in differentiating hepatoblasts (Korz et al., 2001), is expressed in cmp819 mutant livers at 48 hpf, but is not maintained at 96 hpf (100%, n=18 and n=10, respectively; Fig. 1E-H). In addition, group specific component (ge), marking late hepatocyte differentiation (Noël et al., 2010), was not detected in cmp819 mutants (100%, n=15; Fig. 1I-J). Hence, hepatic differentiation fails to progress in cmp819 mutants. Moreover, pan-endodermal expression of the transcription factor foxa1 in cmp819 mutants is similar to wild type at 40 hpf (100%, n=12; Fig. 1K,L), but undetectable at 72 hpf (100%, n=9; Fig. 1M,N). This is due to defects in transcription and not tissue loss, as Tg(XIEef1a1:GFP)825 labels the digestive system in controls and cmp819 mutants at 72 hpf (100%, n=10; Fig. 1O,P). In wild-type embryos, EU labelling of newly synthesised RNA (Jao and Salic, 2008) was detected in all nuclei with higher levels in transcriptionally active sites at 48 hpf (Fig. 1Q-R). These foci were largely absent in cmp819 mutants and overall EU incorporation levels were lower (Fig. 1R-R’), indicating a general defect in RNA transcription. Consistently, actively transcribed genes with a short mRNA half-life, such as those encoding cyclins (Ohtani et al., 1995), represent some of the first genes exhibiting mRNA transcription defects (supplementary material Fig. S4L,M). Altogether, this suggests that cmp819 affects a key factor mediating gene transcription in the differentiating zebrafish liver.

clamped mutations disrupt an orthologue of Ssrp1

Meiotic recombination mapping placed the cmp819 mutation on linkage group 14 in a 340-kb genomic region between markers mindin1 (spon2a) and rs41174000 (primers amplifying a ~160 bp product in an intron of cmp819). F: 5’-GCATAAACGTC-TCTGTGTCG-3’; R: 5’-GCAAGAGAGGATAGAACGACG-3’ (Fig. 2A). Sequencing of five candidate genes within this region revealed a T-to-A base pair substitution at position 309 of ssrp1a in cmp819 mutants, resulting in a premature stop codon and, thus, a truncated protein of 102 amino acids (aa), terminating halfway in the N-terminal Sp16-dimerisation domain (Fig. 2A). The cmp819 lesion was confirmed in mutants by a derived cleaved amplified polymorphic sequences (dCAPs) assay (supplementary material Fig. S1A). Moreover, the mutant u428 failed to complement cmp819 and contained a T-to-G base pair substitution at aa 221 in ssrp1a (R. Young, H. Stickney, T. Hawkins, F. Cavodeassi, G. Gestri, S. Wilson and E.A.O., unpublished; Fig. 2A). Both cmp mutations are predicted to produce functionally inactive proteins, compromising FACT complex formation and function (Keller and Lu, 2002).

Knockdown of Srp1a by injection of morpholino antisense oligonucleotides (MOs) into Tg(XIEef1a1:GFP)825 embryos phenocopied cmp819, with ~80% of embryos showing digestive system hypoplasia (n=9) and 90% displaying smaller eyes, head and fins (n=126; Fig. 2B-G). Consistent with the RNA transcription defects in cmp mutants, previous studies implicated Srp1 function in transcription (Orphanides et al., 1999; Belotserkovskaya et al., 2003; Saunders et al., 2003; Bai et al.,...
Based on these validations, the cmp mutants are referred to as ssrp1as819 and ssrp1au428. All experiments were performed with ssrp1as819, as both alleles display comparable phenotypes.

ssrp1a is expressed ubiquitously in the early embryo and from ~36 hpf in proliferative tissues, such as the liver, eyes and fins (Fig. 2H-L). Quantitative RT-PCR (qPCR) analysis revealed high maternal ssrp1a mRNA levels (2.5 hpf), whereas zygotic expression is much lower (>3 hpf; Fig. 2M). Inhibiting translation of maternal ssrp1a mRNA by MO injection into Tg(XIEef1a1:GFP)s854 embryos does not produce stronger phenotypes compared with ssrp1as819 mutants (Fig. 2B-G), suggesting that maternal Ssrp1a protein compensates for Ssrp1a loss during early development. Alternatively, the zebrafish Ssrp1 parologue Ssrp1b (81% identity)
might act redundantly (supplementary material Fig. S1B), as it is expressed throughout development, albeit at up to 98% lower levels than ssrp1a (Fig. 2M).

**Ssrp1a promotes cell cycle progression in the digestive system and eye**

Hepatic growth arrests in ssrp1a<sup>ss819</sup> mutants at ~48 hpf. BrdU incorporation experiments revealed that at 36 hpf prolifération rates in ssrp1<sup>P1810</sup> mutants (n=10) are similar to those in controls (n=13), but are reduced by ~10% at 38 hpf and by 87% at 49 hpf (n≥10; Fig. 3A-C). This indicates that Ssrp1a promotes hepatic cell divisions. To determine the Ssrp1a-dependent cell cycle phase, mutant and sibling livers were analysed in the transgenic cecyl background labelling S/G2/M phase cells in green and G1 phase cells in red (Sugiyama et al., 2009). At 38 hpf, 55-60% hepatoblasts are found in G1 phase and ~40% in S/G2/M phase in controls and ssrp1a<sup>ss819</sup> mutants (Fig. 3D,E,H). This distribution changes significantly in ssrp1a<sup>P1810</sup> mutants by 48 hpf, with 30% of cells in G1 phase and 62% in S/G2/M phase (n≥7; Fig. 3F-H), indicating that Ssrp1a-deficient hepatoblasts accumulate in S/G2/M phase. Next, we examined single cell suspensions from the foregut domain of Tg(XIeef1a1:GFP)<sup>g854</sup> embryos using FACS. Measuring the DNA content at 42-44 hpf revealed 18.5% fewer Ssrp1a-deficient endodermal cells in G1 phase and, conversely, 10.5% more in S phase compared with wild type (Fig. 3I). This indicates that Ssrp1a is required for S phase progression during development, consistent with in vitro results reporting Ssrp1 functions in DNA elongation (Abe et al., 2011).

Our data indicate that Ssrp1a-deficient cells accumulate in S phase, probably owing to more cells entering or stalling in S phase. A role for Ssrp1a during DNA synthesis, rather than at subsequent checkpoints prior to mitosis, is corroborated by fewer cells incorporating BrdU, and the intact, not polyploid appearance of nuclei in ssrp1a<sup>ss819</sup> livers (Fig. 1Q,R). FACT interacts with the minichromosome maintenance (MCM) helicase in initiation and elongation of DNA synthesis (Gambus et al., 2006; Tan et al., 2006) and, consistent with this, cell cycle defects in ssrp1a<sup>P1810</sup> mutants resemble the ones in Mcm5-depleted zebrafish embryos (Ryu et al., 2005). Intriguingly, Spt16-depleted yeast exhibits a G1 phase delay, pointing to specific functions of either FACT component (Morillo-Huesca et al., 2010). Similarly, human SSRP1 and SPT16 (SUPT16H) can regulate largely overlapping, but also distinct targets (Li et al., 2007).

To determine the specificity of different chromatin-remodelling factors on organ growth, we examined hdac<sup>P456</sup> mutants exhibiting similar liver size defects (Noël et al., 2008). Hdac1 mediates chromatin compaction and cell cycle progression from G<sub>1</sub> to S phase (Yamaguchi et al., 2010), raising the possibility that its loss might counterbalance ssrp1a<sup>P1810</sup> phenotypes. cecyl-based cell cycle analysis at 60 hpf showed that in hdac<sup>P456</sup> mutants 68% of cells are in G<sub>1</sub> phase and 15% in S/G2/M phase (supplementary material Fig. S2A-E), suggesting that Hdac1 promotes cell cycle progression in liver organogenesis. Ssrp1a knockdown in hdac<sup>P456</sup> mutants partially rescues both individual phenotypes, with 26% cells in S/G2/M phase and 55% in G<sub>1</sub> phase (supplementary material Fig. S2A-E), whereas neither organ size, nor hepatic gene expression improves (supplementary material Fig. S2F; data not shown). This is unexpected because in yeast impaired Ssrp1/Pob3 and Hdac/Rpd3(L) functions partially rescue gene transcription and cell viability compared with sole loss of Pob3 (Formosa et al., 2001). This suggests that both factors control different targets mediating cell viability in yeast and zebrafish liver growth.

**Fig. 3. Ssrp1a promotes cell cycle progression. (A-C) BrdU incorporation is reduced in ssrp1a<sup>ss819</sup> mutant livers from 38 hpf onwards (outlined). Error bars represent s.d. (D-G) Transgenic cecyl expression marks G<sub>1</sub> phase cells in red and S/G2/M phase cells in green. (H) Quantification of hepatoblast proliferation shows a reversed distribution in controls and ssrp1a<sup>ss819</sup> mutants at 48 hpf. (I) FACS analysis of 42-44 hpf foregut endoderm shows an increase of cells in S phase and a decrease of those in G<sub>1</sub> phase in ssrp1a<sup>ss819</sup> mutants. A-B,D-G are confocal projections of ventral views; all anterior to the top. *P<0.05, **P<0.0005, determined by unpaired Student’s t-test.**

Compared with the liver, Ssrp1a is required earlier in retina differentiation and proliferation (~36 hpf; supplementary material Fig. S3), indicating similar, but temporally distinct, requirements for zygotic Ssrp1a in a tissue-specific manner. Liver and eye progenitors lacking Ssrp1a undergo apoptosis following S phase defects (supplementary material Fig. S3K-M and Fig. S4). Thus, in ssrp1a<sup>ss819</sup> mutants, incomplete DNA synthesis may trigger a DNA-damage response. Indeed, increased Tp53-target gene expression (Vogelstein et al., 2000) (supplementary material Fig. S4H-K) and a partial rescue of cell death in ssrp1a:tp53 mutants (supplementary material Fig. S4F,G) showed that Tp53-dependent and -independent signalling is activated in Ssrp1a-deficient embryos. Human FACT activates TP53 following DNA damage (Keller et al., 2001) and in Ssrp1-deficient mice apoptosis is solely mediated by Tp53-independent pathways (Cao et al., 2003). By contrast, our data in zebrafish suggest that cell death is not mediated exclusively via Tp53, but might include alternative pathways, highlighting the complexity of the molecular mechanisms following replication stress in different vertebrates.
Full-length ssrp1a and ssrp1b, as well as ssrp1a<sup>1-518</sup>, can compensate for the loss of Drosophila Ssrp

To elucidate the role of the HMGB domain, we performed cross-species rescue experiments using the Drosophila eye. The fly genome has one Ssrp gene (Shimozuma et al., 2003), which encodes an HMGB-containing protein, similar to zebrafish Ssrp1a and Ssrp1b (supplementary material Fig. S1). Knockdown of Ssrp in the fly eye using a UAS-Ssrp<sup>RNAi</sup> transgene caused severe defects (Fig. 4D-F); supplementary material Fig. S6B,B’). Male pharate adults failed to eclose, and lacked eyes, antennae and head capsules, whereas 5.5% of females hatched, and displayed partial head capsules with absent or small eyes. Consistently, eye-antennal imaginal discs of third instar larvae were significantly smaller. Within the eye field, probably because of incomplete knockdown, few cells were phospho-Histone H3 (PH3) positive, and thus mitotically active, and few expressed Elav and mAb24B10 as indicators of photoreceptor (R-cell) differentiation. Upon overexpression of zebrafish full-length ssrp1b, full-length ssrp1a, ssrp1a<sup>6819</sup> or HMGB-deficient ssrp1a<sup>1-518</sup>, males and females hatched with fully developed heads and eyes, and proliferation and differentiation of eye-antennal imaginal discs proceeded normally (supplementary material Figs S5-S7 and Table S1). Strikingly, when Drosophila Ssrp<sup>RNAi</sup> was co-expressed with zebrafish ssrp1b, ssrp1a or ssrp1a<sup>1-518</sup>, the defects caused by Ssrp knockdown were substantially rescued (Fig. 4G-L,P-R); supplementary material Figs S6, S7). The majority of adult females and males hatched and displayed normal heads and eyes (supplementary material Table S1). Adult eyes with a regular array of ommatidia and eye-imaginal discs were indistinguishable from controls. Hence, zebrasfish ssrp1b, ssrp1a and ssrp1a<sup>1-518</sup> can substitute for Drosophila Ssrp. By contrast, truncated ssrp1a<sup>819</sup> did not rescue Drosophila Ssrp knockdown phenotypes (Fig. 4M-O); supplementary material Fig. S6H,H’ and Table S1), indicating that it is neither functional nor acts as a dominant-negative fragment. Importantly, these findings argue that in zebrafish, Ssrp1b could perform all Ssrp1 functions, but probably fails to replace zygotic Ssrp1a owing to low expression levels. Moreover, because Ssrp1a<sup>1-518</sup> can replace Drosophila Ssrp, our data indicate that the C-terminal HMGB-domain is dispensable <i>in vivo</i>. This is consistent with similar observations <i>in vitro</i> (Abe et al., 2011), and the fact that the yeast homologue Pob3 lacking an endogenous HMGB-domain interacts with the HMGB proteins Nhp6a and Nph6b (Wittmeyer et al., 1999). Also, other proteins can compensate for their function, as yeast lacking both factors are viable (Costigan et al., 1994). Likewise, Ssrp1<sup>1-518</sup> could interact with other HMGB proteins to carry out FACT functions, as zebrafish and Drosophila genomes each contain at least four HMGB-containing polypeptides (Ragab et al., 2006) (Ensembl Zv9; http://www.ensembl.org/ Danio_rerio/Info/Index). Our <i>in vivo</i> findings suggest that, despite the presence of an endogenous HMGB-domain, Drosophila, and probably metazoans in general, have maintained the molecular components for Pob3-like FACT function. Hence, the basic mechanism of histone reorganisation between unicellular and multicellular organisms may be conserved independently of gene fusion/separation events associated with Ssrp1 evolution.

Ssrp1a has essential functions in basic cellular processes, including DNA synthesis and gene transcription. We propose that

![Fig. 4. ssrp1b, ssrp1a and ssrp1a<sup>1-518</sup> substitute Drosophila Ssrp function in the eye.](image-url)
the spatiotemporal regulation of ssrplA expression and the aforementioned functions are pivotal for achieving the appropriate rate of cell division and growth of each tissue, ensuring the correct shape, size and organ proportions in the developing embryo. Three lines of evidence indicate that maternal and zygotic Ssrp1a function is specific rather than ubiquitous in zebrafish embryogenesis. First, the onset of zygotic defects in ssrplA mutants is tissue specific. Phenotypes in the eye precede those in the liver but at the same stage no phenotypes are apparent in the somites, despite an earlier phase of extensive proliferation (Sugiyama et al., 2009), suggesting that these dynamics cannot be explained solely by protein turnover associated with replication. This is consistent with specific requirements for Ssrp1a in plant development (Lolas et al., 2010). Second, zygotic ssrplA-expression domains are dynamic over time and often spatially restricted, including presumptive progenitor populations. This is in line with histone chaperones performing functions central to the properties of progenitor populations. Indeed, zebrafish with germ cells lacking maternal and zygotic Ssrp1a develop into sterile males, indicative of essential Ssrp1a functions in germ cell formation (K.K. and V.M. and Reinberg, 2003). The high-mobility-group box protein SSRP1/T160 is essential for cell viability in day 3.5 mouse embryos. Mol. Cell. Biol. 23, 5301-5307.


