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RESEARCH REPORT

RPSA, a candidate gene for isolated congenital asplenia, is required for pre-rRNA processing and spleen formation in *Xenopus*

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ABSTRACT

A growing number of tissue-specific inherited disorders are associated with impaired ribosome production, despite the universal requirement for ribosome function. Recently, mutations in RPSA, a protein component of the small ribosomal subunit, were discovered to underlie approximately half of all isolated congenital asplenia cases. However, the mechanisms by which mutations in this ribosome biogenesis factor lead specifically to spleen agenesis remain unknown, in part due to the lack of a suitable animal model for study. Here we reveal that RPSA is required for normal spleen development in the frog, *Xenopus tropicalis*. Depletion of Rpsa in early embryonic development disrupts pre-rRNA processing and ribosome biogenesis, and impairs expression of the key spleen patterning genes *nkx2-5*, *bapx1* and *pod1* in the spleen anlage. Importantly, we also show that whereas injection of human RPSA mRNA can rescue both pre-rRNA processing and spleen patterning, injection of human mRNA bearing a common disease-associated mutation cannot. Together, we present the first animal model of RPSA-mediated asplenia and reveal a crucial requirement for RPSA in pre-rRNA processing and molecular patterning during early *Xenopus* development.

KEY WORDS: Asplenia, RPSA, Ribosomopathy, *Xenopus*, rRNA processing

INTRODUCTION

As the molecular machines responsible for producing proteins, ribosomes are ubiquitously essential for life. Indeed, a single mammalian cell can contain 10 million individual ribosomes and devote 70% of total cellular transcription to ribosome production (Freed et al., 2010; Warner et al., 2001). Ribosome biogenesis is an extraordinarily complex process that occurs in multiple cellular compartments and requires the coordinated action of more than 200 distinct ribosome biogenesis factors, all three RNA polymerases and 75 small nucleolar RNAs (Armistead and Triggs-Raine, 2014; Freed et al., 2010; Gallagher et al., 2004; Granneman and Baserga, 2004; Henras et al., 2008; Woolford and Baserga, 2013). Production starts in the nucleolus, with transcription of pre-ribosomal RNAs (pre-rRNA), and continues through a series of carefully regulated cleavages and ribonucleoparticle assembly steps to ultimately produce the large 60S ribonucleoprotein subunit (LSU, an intricate complex of 46 ribosomal proteins and the 28S, 5.8S and 5S rRNAs) and the small 40S subunit (SSU, 33 ribosomal proteins and the 18S rRNA) (Armistead and Triggs-Raine, 2014; Henras et al., 2008; Sondalle and Baserga, 2014). Along with cytoplasmic translation factors, the LSU and SSU form a functional ribosome.

It is not surprising that defects in ribosome production or function are detrimental to health. Indeed, mutations that affect ribosome biogenesis have now been identified in numerous human congenital disorders (ribosomopathies) and multiple animal models are available for study (Armistead et al., 2009; Armistead and Triggs-Raine, 2014; Boocock et al., 2003; Dauwerse et al., 2011; Devlin et al., 2010; Dixon et al., 1997; Ebert et al., 2008; McGowan et al., 2008; Mirabello et al., 2017, 2014; Nousbeck et al., 2008; Uechi et al., 2008; Wilkins et al., 2013; Zhao et al., 2014). However, these mutations produce an intriguingly heterogeneous collection of clinical phenotypes that are surprisingly tissue specific given the ubiquitous requirement for ribosomes. For example, inherited mutations in any of at least 19 proteins, many of them ribosomal, cause Diamond-Blackfan anemia, which is associated with bone marrow failure, craniofacial, limb and growth defects (Boria et al., 2010; Farrar et al., 2014; Gazda et al., 2012, 2008; Landowski et al., 2013; Mirabello et al., 2017; Wang et al., 2015). TCOF1 mutations are primarily associated with craniofacial abnormalities (Treacher-Collins syndrome) (Dixon et al., 1997, 2006; Sondalle and Baserga, 2014), whereas mutations in another ribosome biogenesis factor, UTP4, cause North American Indian childhood cirrhosis, a specific childhood liver failure syndrome (Chagnon et al., 2002; Freed and Baserga, 2010). Ribosomopathies are, therefore, clinically distinct and difficult to treat (Armistead and Triggs-Raine, 2014; Danilova and Gazda, 2015). Investigating the etiology of these tissue-specific phenotypes will transform our understanding of ribosomopathies and provide a crucial step towards improving diagnosis and treatment.

Isolated congenital asplenia (ICA, OMIM #271400) is a rare and often fatal developmental anomaly, which has recently been identified as a ribosomopathy (Bolze et al., 2013). Characterized by the absence or severe hypoplasia of the spleen at birth in otherwise healthy individuals, ICA is a form of primary immunodeficiency that leaves patients susceptible to bacterial infections (Gilbert et al., 2002; Lindor et al., 1995; Mahlaoui et al., 2011). While antibiotic prophylaxis can decrease mortality, ICA often goes undiagnosed because of the absence of an external phenotype and patients typically succumb to sepsis in early childhood.

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Spleen development remains poorly understood. Studies in humans and in animal models demonstrate that the spleen anlage forms as a condensation of mesenchymal progenitor cells within the left dorsal mesogastrium (Brendolan et al., 2007; Patterson et al., 2000; Sty and Conway, 1985; Vellguth et al., 1985). These progenitors proliferate and interact with contributing endothelial and hematopoietic cells to ultimately produce a functional spleen. Previous work has revealed a gene regulatory network, including *Nkx2-5*, *Bapx1* (also known as *Nkx3-2*), *Pbx1*, *Hox11* (also known as *Tlx1*) and *Pod1* (also known as *Tcf21*), that regulates this process (Brendolan et al., 2005; Burn et al., 2008; Herzer et al., 1999; Koss et al., 2012; Lettice et al., 1999; Lu et al., 2000; Pabst et al., 1999; Patterson et al., 2000; Roberts et al., 1994). While laterality defects can also produce syndromic forms of asplenia (Burton et al., 2014; Kothari, 2014), ICA is considered to be distinct, as it occurs in isolation.

Multiple heterozygous mutations in the ribosomal SSU protein RPSA have been identified in ICA patients, although the penetrance appears incomplete (Bolze et al., 2018, 2013). RPSA is a highly conserved protein that is required for protein synthesis and maturation of the 18S rRNA component of the 40S subunit (Auth and Brawerman, 1992; Malygin et al., 2011; Nelson et al., 2008; O'Donohue et al., 2010). In vertebrates the C-terminal region of RPSA has evolved an additional role as a receptor for laminin (Ardini et al., 1998; Lesot et al., 1983; Nelson et al., 2008; Rao et al., 1983; Terranova et al., 1983). Interestingly, no pre-rRNA processing defects were detected in activated lymphocytes from ICA patients, and heterozygous null *RPSA* mutant mice do not exhibit asplenia (Bolze et al., 2013). Thus, the mechanisms by which mutations in RPSA lead to spleen agenesis remain undefined, and there has been no animal model of the human disease.

Here we reveal that *rpsa* is required for normal spleen development in the frog, *Xenopus tropicalis*. Depletion of Rpsa disrupts pre-rRNA processing and ribosome biogenesis, and impairs expression of the key spleen patterning genes *nkx2-5*, *pbx1* and *pod1* in the spleen anlage. Importantly, we also show that whereas injection of human RPSA mRNA can rescue both pre-rRNA processing and spleen patterning, mRNA containing the most common disease mutation cannot. Altogether, we present the first animal model of RPSA-mediated asplenia and reveal a crucial requirement for RPSA in pre-rRNA processing and molecular patterning of the spleen during vertebrate development.

RESULTS AND DISCUSSION

Expression of *rpsa* is dynamically regulated during development

As an initial step in our investigation, we examined *rpsa* mRNA expression during *X. tropicalis* embryogenesis. Despite a global requirement for ribosome biogenesis, we observed tissue-specific enrichment of *rpsa* transcripts. No *rpsa* expression was detectable by *in situ* hybridization of labeled antisense probes at pregastrulation stages (Fig. 1A), suggesting that *Xenopus* embryos do not inherit large amounts of maternal *rpsa* mRNA. At stage 16, expression was strongly detected throughout the developing neural folds. Transcripts were robustly detected in posterior regions of the neural tube at stage 24, but appeared relatively weak within anterior regions and the brain. Expression of *rpsa* was also evident in migrating neural crest at this stage (Fig. 1A). This association of *rpsa* expression with the developing neural tube, brain, neural crest and intersomitic spaces was maintained at stage 28. Importantly, expression was also diffusely detected in the anterior lateral mesoderm from which the spleen forms, and within the ventral blood islands and mesoderm (Fig. 1A).

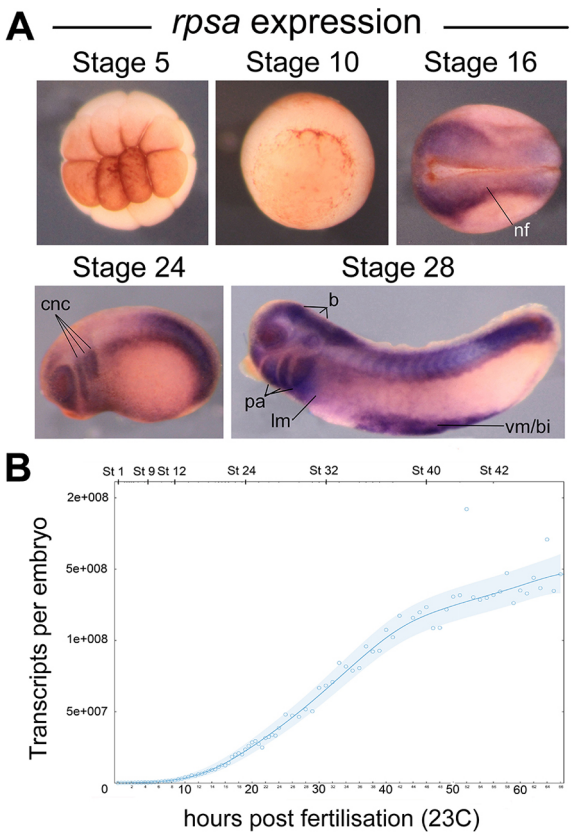

Fig. 1. *rpsa* is dynamically expressed in development. (A,B) Both *in situ* hybridization (A) and ultra-high temporal resolution RNA-seq analysis (B) reveal low levels of *rpsa* transcripts prior to stage 12 in *X. tropicalis*. Expression increases throughout subsequent stages and is primarily associated with the developing neural folds (nf), brain (b), cranial neural crest (cnc) and ventral mesoderm/blood islands (vm/bl). Expression is also diffusely detected in the lateral mesoderm (lm) where the spleen will form. pa, pharyngeal arches.

To quantitatively analyze embryonic *rpsa* expression dynamics, we used our previously published ultra-high temporal resolution RNA-seq data set (Owens et al., 2016). This resource allows precise measurement of absolute transcripts per embryo and visualization of transcript kinetics for the entire transcriptome during *Xenopus* development. Consistent with our *in situ* results, these quantitative data revealed extremely low levels of *rpsa* prior to gastrulation and a steady increase in transcription from neurulation onwards (Fig. 1B).

Spleen development is impaired in *rpsa* morphants

We used translation ($rpsa^{ATG}$) and splice ($rpsa^{Splice}$) blocking morpholino oligonucleotides (MO) to deplete Rpsa and investigate its requirements in development. With the exception of a mild reduction in anterior-posterior length, *rpsa* morphants appear comparable with controls at stage 45 (Fig. S1A) and are able to swim and feed normally. However, morphants fail to increase in size and become significantly less active post stage 45. By stage 48, 50% of morphants develop a mild edema, and all die within 12 days of fertilization. Addition of gentamycin failed to improve survival, which suggested that infection is not a major cause of death.

As late stage lethality prevented direct examination of adult spleen anatomy, we instead assayed the expression of crucial splenic genes at earlier stages of development. The transcription factors that are encoded by the genes *bapx1*, *nkx2-5* and *pod1* are essential for spleen formation (Brendolan et al., 2007; Koss et al., 2012; Lettice

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et al., 1999; Lu et al., 2000; Patterson et al., 2000; Quaggin et al., 1999). Indeed, in human ICA patients, homozygous missense mutations in *NKX2-5* have been identified (Koss et al., 2012). Knockdown of Rpsa results in severe reduction or loss of *nkx2-5*, *bapx1* and *pod1* expression in the developing spleen of stage 38 embryos, whereas expression remains relatively unaltered in other regions (Fig. 2, Fig. S2). Importantly, this phenotype persisted at later stages of development (stage 40, Fig. S2B), revealing that it is not caused by a developmental delay. Together, these observations demonstrate that *rpsa* is required for early spleen formation in *Xenopus*. As 2 ng and 4 ng of *rpsa* MO produced a similar *nkx2-5* phenotype (Fig. 2), we injected 2 ng in all subsequent experiments.

As human mutations in RPSA cause ICA, we further investigated the specificity of the phenotype by examining the development of other structures. Formation of the craniofacial cartilages, eyes, heart and gut appeared grossly normal in stage 45 morphants (Fig. S1A,B). We noted that *nkx2-5* expression is relatively unaffected in its cardiac domain, despite being lost in the spleen of *rpsa* morphants at stages 38 and 40 (Fig. 2, Fig. 3, Fig. S2). Expression of the gene encoding the transcription factor Nkx2-1 also appeared normal in the developing lungs, forebrain and olfactory region of morphants at stage 38 (Fig. S1C). Expression of *hex* (a key regulator of endoderm organogenesis) and *pax2* (in the brain, otic placode and kidney) were similarly unaffected by Rpsa depletion at this stage (Fig. S1C). As congenital asplenia is commonly associated with laterality syndromes, we also examined expression of *pitx2c*, a key asymmetrically expressed gene in left-right development. No evidence of a laterality defect was detected in stage 28 morphants (Fig. S1D). Together, these results demonstrate that Rpsa depletion is affecting spleen patterning in the early embryo, recapitulating the core of the human patient phenotype.

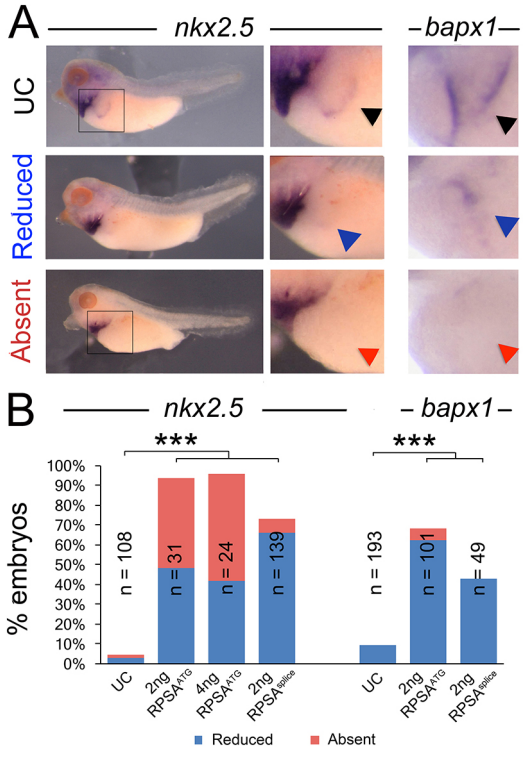

Fig. 2. Depletion of Rpsa using either a translation (Rpsa^{ATG}) or splice (Rpsa^{Splice}) blocking morpholino impairs expression of *nkx2-5* and *bapx1* in the spleen of stage 38 *rpsa* morphants. (A) Representative images of the expression phenotype. Middle and right panels are magnifications of the spleen region marked by a box in the left panel. Black arrowheads indicate normal expression. Blue arrowheads indicate reduced expression. Red arrowheads indicate lack of expression. (B) The percentage of embryos with reduced or absent *nkx2-5* or *bapx1* expression in the developing spleen following *rpsa* depletion. ***$P \leq 0.0001$ (two-tailed Fisher's exact test).

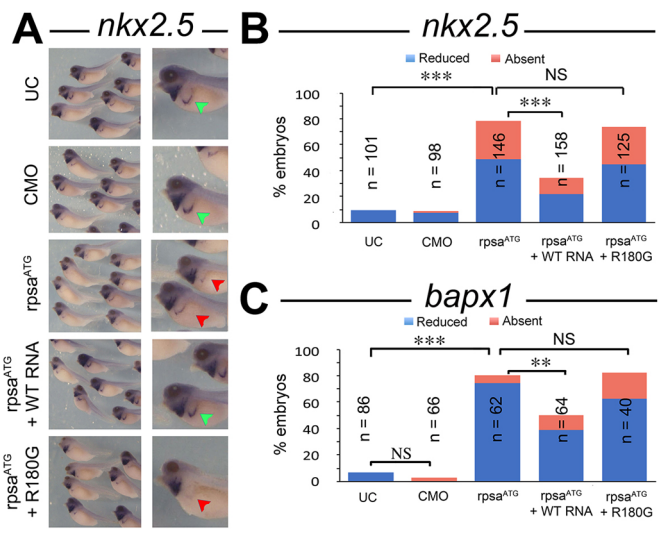

Fig. 3. The human R180G mutation is detrimental to function. (A) Representative images of *nkx2-5* expression in the spleen anlage of stage 38 embryos. Depletion of Rpsa causes a severe reduction of *nkx2-5* expression, which can be ameliorated by injection of human WT RPSA mRNA but not by injection of R180G mutated mRNA. Green arrowheads highlight robust expression; red arrowheads indicate reduced expression. (B,C) The percentage of embryos with reduced or absent *nkx2-5* (B) or *bapx1* (C) expression. **P<0.001, ***P<0.0001 (two-tailed Fisher's exact test). CMO, control morpholino; rpsa^{ATG}, translation-blocking MO; NS, not significant; UC, uninjected control.

A human disease mutation disrupts RPSA function in *Xenopus*

In ICA patients a recurrent missense mutation, R180G, was identified (Bolze et al., 2013). This position is highly conserved (Ardini et al., 1998) (Fig. S3A); however, whether the patient allele is deleterious was previously undefined. Therefore, we first examined the structure of RPSA from a cryo-electron microscopy (cryo-EM) reconstruction of the 80S human ribosome to determine the molecular consequences of the R180G mutation. Our structural analysis revealed that arginine 180 (R180) in RPSA has predicted polar contacts with glutamate 181 (E181) and aspartate 14 (D14) (Fig. S3B). Mutation of residue 180 to glycine (R180G) is predicted to abolish these interactions, likely destabilizing the protein's tertiary structure (Fig. S3C).

To test this functionally, we examined this variant using our *Xenopus* model. Importantly, co-injection of full-length human *RPSA* mRNA rescued expression of both *nkx2-5* and *bapx1* in the spleen of morphants at stage 38 (Fig. 3, Fig. S2), demonstrating the specificity of our MO-mediated knockdown strategy. Injection of human mRNA carrying the R180G mutation was unable to rescue expression of either gene (Fig. 3, Fig. S2), suggesting that the disease mutation impairs the RPSA function required for spleen development.

Pre-rRNA processing is impaired in *rpsa* morphants

RPSA has previously been implicated in late maturation of the 18S rRNA in cell culture (O'Donohue et al., 2010). Using an antisense oligonucleotide probe targeting the internal transcribed spacer (ITS1) of *X. tropicalis* (probe c, Fig. 4A), we investigated the

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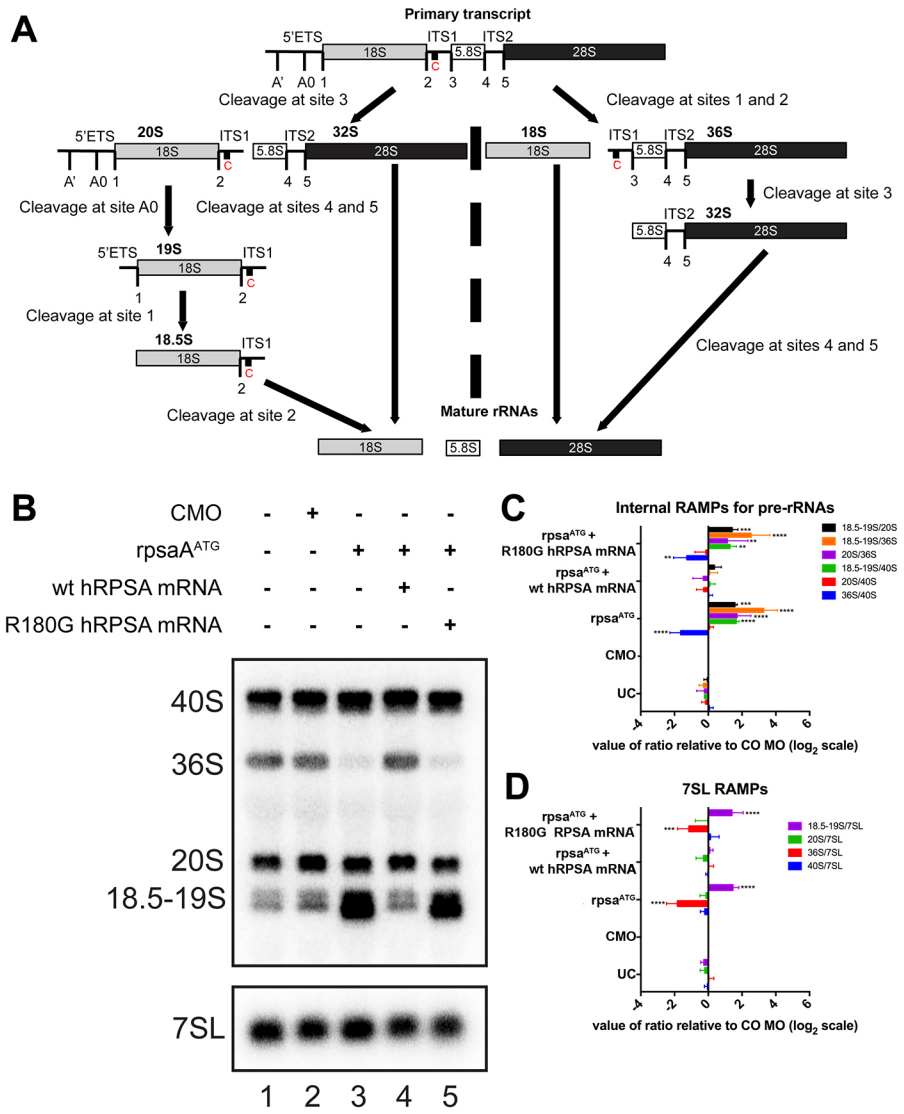

Fig. 4. Rpsa is required for pre-rRNA processing. (A) Diagram of pre-rRNA processing pathways in *X. tropicalis*. The primary transcript pre-rRNA undergoes several nucleolytic cleavages to produce the mature 18S, 5.8S and 28S rRNAs. c (red), position of the northern blot probe; ETS, external transcribed spacers; vertical lines indicate cleavage sites. (B-D) Depletion of Rpsa causes pre-rRNA processing defects that are rescued by WT human RPSA (hRPSA) but not by ICA-mutated RPSA mRNA (R180G). Stage 38 *X. tropicalis* pre-rRNAs isolated from pools of 10-15 whole embryos were detected by northern blotting (B). The 7SL RNA was used as a loading control. +, injection; –, no injection. (C) RAMP quantitation of the ratios of pre-rRNAs relative to each other. (D) RAMP quantitation of the pre-rRNAs relative to the 7SL loading control. Data are mean+s.d. for four independent biological replicates. $^{**}P\leq0.01$, $^{***}P\leq0.001$, $^{****}P\leq0.0001$ (two-way ANOVA with Tukey's multiple comparisons test). CMO, control morpholino; rpsa^{ATG}, translation-blocking MO; UC, uninjected control.

requirement for *rpsa* in pre-rRNA processing during vertebrate development. Probe c revealed an accumulation of the 18.5S and 19S pre-rRNAs in stage 38 morphants, consistent with a defect in later maturation steps of the 18S rRNA (Fig. 4B, lane 3, and 4C,D). Unexpectedly, Rpsa depletion also caused a striking reduction in 36S pre-rRNA levels (Fig. 4B, lane 3, and 4C,D). The reduced levels of the 36S pre-rRNAs, concomitant with the accumulation of the 18.5S and 19S pre-rRNAs, are consistent with a requirement for Rpsa in pre-rRNA cleavage at site 2 (Fig. 4A). Therefore, our results demonstrate a crucial requirement for Rpsa in embryonic 18S rRNA biogenesis and reveal a role for Rpsa in production of the 36S pre-rRNA (Fig. 4).

To confirm that these cleavage defects were specific to Rpsa depletion, we co-injected the human *RPSA* mRNA, which rescued all the processing defects that were observed in *rpsa* morphants (Fig. 4B, lane 4, and 4C). Conversely, injection of the R180G ICA mutant mRNA did not rescue pre-rRNA processing (Fig. 4B, lane 5, and 4C, D). Together, these data confirm that RPSA has an evolutionarily conserved function in ribosome biogenesis during development, which is disrupted by the R180G ICA disease mutation.

RPSA has dual functions in the cell. Its highly conserved N-terminal region plays a critical role in formation of the 40S SSU, while its C-terminal region has evolved a relatively new role as a receptor for laminin (Ardini et al., 1998; Auth and Brawerman, 1992; Lesot et al., 1983; Malygin et al., 2011; Nelson et al., 2008; O'Donohue et al., 2010; Rao et al., 1983; Terranova et al., 1983). Thus, the precise mechanism through which mutations in RPSA cause ICA remains undefined. However, while Bolze et al. failed to detect pre-rRNA processing defects in ICA patient lymphocytes, three of the seven identified patient mutations occurred in regions of the RPSA N-terminus that are associated exclusively with ribosome biogenesis (Ardini et al., 1998; Bolze et al., 2013). Furthermore, all of the identified mutations, including R180G, affected residues that are conserved from mammals to lower eukaryotes, such as *Saccharomyces cerevisiae* and *Neurospora crassa,* which predate the advent of laminin receptor function, suggesting that RPSA's ribosomal function is essential to spleen development. Our observation that human *RPSA* mRNA can rescue both pre-rRNA processing and spleen patterning, whereas the R180G disease mutation cannot, demonstrates that the human mutation disrupts a ribosomal function of RPSA and supports the hypothesis that impaired ribosome production underlies ICA.

How mutations in the ubiquitous production of ribosomes lead to tissue-specific phenotypes, such as spleen agenesis, remains an

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intriguing issue. One possibility is that particular cell populations are more sensitive to reduced rates of mRNA translation (Green et al., 2000). The cranial neural crest, for example, is particularly sensitive to impaired ribosome production and depletion of several distinct ribosome biogenesis factors can trigger P53-mediated cell death (Amsterdam et al., 2004; Calo et al., 2018; Dixon et al., 2006; Griffin et al., 2015; Hölzel et al., 2010; Jones et al., 2008; Valdez et al., 2004; Wilkins et al., 2013; Zhao et al., 2014). However, this sensitivity alone seems unlikely to explain the distinct tissue-specific phenotypes of ribosomopathies. For example, why are such cranial neural crest malformations not observed in all ribosomopathies? Or why does the bone marrow failure that characterizes Diamond Blackfan anemia not occur in Treacher Collins syndrome? Another possible explanation is that ribosomes may be 'specialized' to have unique properties or preferentially translate particular mRNAs in diverse cell populations. Indeed, support for the specialized ribosome hypothesis has been growing, with observations of tissue-specific differences in ribosomal protein expression (Bortoluzzi et al., 2001; Green et al., 2000; Robson et al., 2016; Sahin et al., 2005; Wong et al., 2014; Zhao et al., 2014), and context-dependent selective mRNA translation by particular ribosomal proteins (Dinman, 2016; Ferretti et al., 2017; Horos et al., 2012; Kondrashov et al., 2011; McCann and Baserga, 2013; Segev and Gerst, 2018; Shi and Barna, 2015; Shi et al., 2017; Xue and Barna, 2012). Our study reveals that Rpsa depletion preferentially affects early patterning of the spleen, and places *rpsa* upstream of the known genetic network that regulates spleen formation. However, it remains for future studies to determine whether this loss of patterning occurs through reduced translation of spleen regulatory mRNAs, increased local cell death or a currently unknown mechanism.

It is important to note that *rpsa* morphants have additional defects at later stages of development and, as such, the asplenia does not occur in isolation in our model. These additional phenotypes most likely reflect the level of Rpsa knockdown present in morphants. Rpsa is required in all cells and the degree of developmental defect appears to reflect dose. For example, Rpsa null mice die at E3.5, whereas heterozygotes are completely normal. Most human patients carrying deleterious heterozygous mutations develop ICA (Bolze et al., 2018, 2013). Our morphants most likely have an intermediate reduction in Rpsa levels that results in additional defects at later stages of development. However, reduced expression of three genes that are required for spleen formation in *rpsa* morphants, together with normal embryonic morphology and unaffected expression of non-spleen genes, supports the hypothesis that the spleen is particularly sensitive to reduced levels of RPSA.

In this study, we have investigated Rpsa requirements in spleen development. We present the first animal model of Rpsa-mediated asplenia and demonstrate an evolutionarily conserved requirement for *rpsa* in pre-rRNA processing and molecular specification of the spleen during embryogenesis. The genetic regulation of spleen development and the causes of spleen anomalies, ICA in particular, have long remained unclear. Initial attempts to study the mechanism of RPSA-mediated asplenia have been hampered by the lack of an appropriate animal model (heterozygous null *Rpsa* mice develop normal spleens, while homozygous null mutants die in early development) (Bolze et al., 2013). Furthermore, although mouse models have sometimes been informative in the study of ribosomopathies, the phenotype is often very sensitive to genetic background, which complicates analysis (Dixon et al., 2006; Jones et al., 2008). Here, we used MOs, which allow for efficient and tractable protein depletion, to produce a novel animal model of *RPSA*-mediated asplenia. Our work places *rpsa* in the gene regulatory network that orchestrates spleen development, identifies its function in pre-rRNA processing and supports a role for impaired ribosome biogenesis in the pathogenesis of ICA. We highlight the utility of *Xenopus* as a model in which to study ribosomopathies and believe that this approach holds value for future studies of human asplenia.

MATERIALS AND METHODS

Xenopus

X. tropicalis were maintained and cared for in our aquatics facility, in accordance with Yale University Institutional Animal Care and Use Committee protocols. Embryos were produced and raised to appropriate stages as previously described (del Viso and Khokha, 2012).

Antisense morpholino knockdown and RNA rescue

Antisense MOs targeting the *rpsa* translational start site (5′-CCGGAC-ATTGTGAGTTACCCCTTT-3′) or standard control morpholinos (CMO) were obtained from Gene Tools and injected at the one-cell stage. A full-length human *RPSA* cDNA was obtained from the hORFeome v8.1 library from the Center for Cancer Systems Biology at Dana-Farber Cancer Institute (HsCD00508936) and subcloned into the pCSDest2 vector using Gateway recombination techniques (Invitrogen). A human R180G RPSA clone was synthesized using the GeneArt strings DNA fragment service of Life Technologies. Capped mRNAs were generated *in vitro* using the mMessage machine kit (Ambion) according to the manufacturer's instructions.

In situ hybridization

Digoxigenin-labeled antisense probes for *rpsa* (TNeu119p20; Genbank: NM_203737.1), *pitx2c* (TNeu083k20; Genbank: CR760278.2), *hex* (TGas075h17; Genbank: CR761571.2) and *pax2* (TNeu062i10; GenBank: CR760264.2) were *in vitro* transcribed with T7 High Yield RNA Synthesis Kit (E2040S, New England Biolabs). A *bapx1* fragment for probe synthesis was produced by PCR (primers 5′-TTGGGATCCATTT-GGGGAGC-3′ and 5′-TGGTGGTTGA- AGCGTCTCTC-3′) and cloned in pCS108. Embryos were fixed in MEMFA (10% 10× MEMFA salt, 10% formaldehyde, 80% water) for 1-2 h at room temperature and dehydrated into 100% ETOH. Whole-mount *in situ* hybridization was performed as described previously (Khokha et al., 2002).

Northern blotting

Pools of ten to 15 embryos were dissolved in TRIzol (Invitrogen) and total RNA was extracted as per the manufacturer's instructions. Northern blot analyses were performed as described previously (Pestov et al., 2008). Then 2 µg or 4 µg of total RNA per sample were separated by gel electrophoresis on a 1% agarose/1.25% formaldehyde gel and then transferred to a nylon membrane (Hybond-XL, GE Healthcare). RNA species were detected by hybridization with a radiolabeled oligonucleotide probe. Oligonucleotide probes were: Probe c, 5′-CAGGTACCCGGGTCGGCCTGCGGCG-3′; 7SL: 5′-CATA- TTGATACCGAACTTAGTGC-3′.

Imaging

Images were taken using a Canon DS126201 camera and Zeiss Discovery V8 SteREO microscope. Optical coherence tomography of the craniofacial cartilages was carried out as previously described (Deniz et al., 2017).

Structural modeling

All structural models of RPSA were generated from a cryo-EM structure of the human 80S ribosome (Protein Data Bank ID: 4UG0) (Khatter et al., 2015) using the PyMOL Molecular Graphics System, version 2.0.6 (Schrödinger). Identification of polar interactions and modeling of mutated residues were also performed using PyMOL.

Quantification and statistical analysis

Each experiment was repeated a minimum of three times. Statistical significance is reported in the figures and legends and was defined as $P<0.05$

(*P<0.01, **P<0.001 and ***P<0.0001). In Figs 2, 3 and Fig. S2 the *in situ* hybridization results were analyzed using a two-tailed Fisher's exact test. In Fig. 4, a phosphorimager (Bio-Rad Personal Molecular Imager) was used to quantify northern blots. Ratio analysis of multiple precursors (RAMP) was performed as described in Wang et al. (2014) and a two-way ANOVA with Tukey's multiple comparisons test for post hoc analysis was used to determine statistical significance.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.N.G., S.J.B., M.K.K.; Methodology: J.N.G., S.B.S., S.J.B., M.K.K.; Validation: J.N.G.; Formal analysis: J.N.G., S.B.S., S.J.B., M.K.K.; Investigation: J.N.G., S.B.S., A.R., E.K.M., G.G., S.S.K., E.D.; Resources: J.N.G., S.J.B., M.K.K.; Writing - original draft: J.N.G.; Writing - review & editing: J.N.G., S.B.S., S.J.B., M.K.K.; Supervision: J.N.G., S.J.B., M.K.K.; Project administration: J.N.G., S.J.B., M.K.K.; Funding acquisition: S.J.B., M.K.K.

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Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.166181.supplemental

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