Mutation of RNA Pol III Subunit rpc2/polr3b Leads to Deficiency of Subunit Rpc11 and Disrupts Zebrafish Digestive Development

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The role of RNA polymerase III (Pol III) in developing vertebrates has not been examined. Here, we identify a causative mutation of the second largest Pol III subunit, polr3b, that disrupts digestive organ development in zebrafish slj (slj) mutants. The slj mutation is a splice-site substitution that causes deletion of a conserved tract of 41 amino acids in the Polr3b protein. Structural considerations predict that the slj Pol3rb deletion might impair its interaction with Polr3k, the ortholog of an essential yeast Pol III subunit, Rpc11, which promotes RNA cleavage and Pol III recycling. We engineered Schizosaccharomyces pombe to carry an Rpc2 deletion comparable to the slj mutation and found that the Pol III recovered from this rpc2Δ yeast had markedly reduced levels of Rpc11p. Remarkably, overexpression of cDNA encoding the zebrafish rpc11 ortholog, polr3k, rescued the exocrine defects in slj mutants, indicating that the slj phenotype is due to deficiency of Rpc11. These data show that functional interactions between Pol III subunits have been conserved during eukaryotic evolution and support the utility of zebrafish as a model vertebrate for analysis of Pol III function.

Introduction

RNA Polymerase III (Pol III) is a 17-subunit complex that is responsible for the transcription of small noncoding RNAs such as transfer RNAs (tRNAs), 5S ribosomal RNA (rRNA), U6 small nuclear RNA (snRNA), 7SL RNA, and others in eukaryotes [1,2]. The two largest subunits, Rpc1 (~160 kDa) and Rpc2 (~130 kDa), are highly homologous to their counterparts in Pol I and Pol II, and together provide a large surface area for interaction with many of the other subunits [2]. Structural analyses of Pol III complexes [3,4], together with two-hybrid analysis [5], have identified multiple subunit interactions (reviewed in [1]). These, together with biochemical and genetic analyses, have led to a model that attributes some of the unique functions of Pol III, including its high processivity, efficient transcription termination and recycling activity, RNA 3' cleavage activity, and interaction with diverse promoters, to specific individual subunits.

Mutational analyses in yeast clearly show that an intact Pol III system is essential for cell growth. The effects of reduced Pol III function are predicted to be broad, including protein synthesis necessary for cell-cycle progression (tRNAs), ribosome biogenesis (5S rRNA), mRNA splicing (U6 snRNA), and membrane targeting of newly translated proteins (7SL RNA). Pol III transcription is tightly regulated during the cell cycle [6] and in response to cellular stress [7]. Recent studies in human cells have also highlighted the roles of oncogenes and tumor suppressors such as Rb [8,9], p53 [9–11], and cMyc [9,12] in controlling the interactions between the transcription factors that bring the Pol III complex to the promoters of its target genes (reviewed in [13,14]). Other proteins, such as MafI [15–18] and the oncogenic kinase CK2 [19–20], can regulate Pol III function through direct interactions with the Pol III complex. Thus, eukaryotic cells have evolved multiple independent mechanisms for regulating Pol III activity.

Given the importance of Pol III for cell growth and proliferation, it is not surprising that it is deregulated in cancers and in cells transformed by viral oncoproteins [13,14]. These findings suggest that it may be possible to disrupt transformed cells by inhibiting Pol III function. It is not known, however, whether Pol III inhibition has deleterious effects in nonproliferating cells of complex multi-

Abbreviations: aa, amino acid; BAC, bacterial artificial chromosome; BrdU, bromodeoxyuridine; dpf, days post-fertilization; hpf, hours post-fertilization; PH3, phospho-Histone H3; Pol III, RNA polymerase III; rRNA, ribosomal RNA; RT-PCR, real-time PCR; slj, slim jim; snRNA, small nuclear RNA; RNA, transfer RNA

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cellular organisms. Here, we describe the positional cloning of a mutation, slim jim (slj) [21–23], that targets polr3b, the zebrafish ortholog of a yeast Pol III subunit gene rpc2, which is highly conserved in eukaryotes. tRNAs and other Pol III transcripts are decreased in slj larvae. Accordingly, the slj mutation has a pronounced effect on the growth and proliferation of progenitor cells in the digestive tract of slj larvae, but surprisingly, no overt effect on the survival of cells in other nonproliferating mutant tissues at the same developmental stage. Also unexpectedly, the slj mutation does not interfere with differentiation of most epithelial lineages in the developing zebrafish intestine, but has a profound effect on epithelial cell morphology [22]. These data surprisingly indicate that specific cell types in the developing fish are differentially sensitive to the slj mutation and suggest that this may be due to different requirements for Pol III activity.

Structural comparisons of yeast Pol II and Pol III suggest that the slj mutation might perturb interaction of Rpc2 with Rpc11, an integral Pol III subunit that exhibits RNA 3′ cleavage activity and is required for efficient transcription recycling by Pol III [24–26]. Supporting this idea, we show that the Rpcllp protein is not present in the Pol III complex purified from S. pombe engineered to carry an Rpc2 deletion mimicking the polr3bΔ allele. Microinjection of an overexpression construct encoding the zebrafish Rpc11 ortholog, Polr3k, suppresses the exocrine defects in slj larvae, indicating that the slj phenotype is due to deficiency of the Polr3b–Polr3k (Rpc2–Rpc11) interaction, and supporting broad conservation of Pol III structure in eukaryotes.

Results

Cell Type–Specific Reduction of Cell Proliferation in the Zebrafish Digestive Organs

The zebrafish slj mutation was recovered in a large-scale mutagenesis screen on the basis of altered intestinal morphology [21]. Compared to wild-type five-day post-fertilization (dpf) larvae, the intestine of 5-dpf slj larvae is small, thin walled, and lacks folds (Figure 1A and 1B). In histological sections, the slj intestinal epithelium appears immature compared with wild-type siblings (Figure 1C and 1D). Development of the exocrine pancreas is also severely affected by the slj mutation [23]. Little, if any, exocrine tissue is visible in histological sections of 5-dpf slj larvae (Figure 1G and 1H). By contrast, the pancreatic islet, which undergoes little or no expansion beyond the first 48 hours post-fertilization (hpf) appears normal in slj (Figure 1G and 1H).

To investigate the underlying cause of this feature of the slj phenotype, we measured slj intestinal and pancreas cell proliferation using bromodeoxyuridine (BrdU) and phospho-Histone H3 (PH3) immunohistochemistry. The BrdU assay revealed a nearly 2-fold reduction in the proportion of S-phase cells within the slj intestinal epithelium (Int; Table 1) and a nearly 5-fold reduction of the proportion of S-phase cells in the developing exocrine pancreas (ExP; Table 1) around the stage when the slj phenotype is first recognizable (72 hpf). By contrast, cell proliferation within the intestinal stroma was not affected by the slj mutation at this stage. Reduced proportion of intestinal epithelial and pancreatic S-phase cells, coupled with the normal percentage of M-phase cells at this stage (as determined by anti-PH3 immunohistochemistry), suggest the slj mutation causes a delay in the G1–S transition within highly proliferative organ progenitor cells. Consistent with this idea, the size of other highly proliferative tissues, such as the liver, retina, and terminal branchial arches, was also reduced in slj larvae (Figure 1E and 1F, and unpublished data).

Causative Mutation of Zebrafish polr3b in Zebrafish slj Mutants

To gain a better understanding of the cause of the proliferative defects associated with the slj phenotype, a positional cloning strategy was used to identify the targeted gene. Using bulk segregant analyses, we first identified a marker on Chromosome 18 linked to the slj locus (M. Mohideen, M. Fishman, and M. Pack; unpublished data). Mapping of subsequent markers identified a critical region surrounding the slj locus as described in Materials and Methods and Figure 2A. Ultimately, a bacterial artificial chromosome (BAC, zk103i16) spanning two flanking simple-sequence repeat markers (z15417 and z6098) was identified. Microinjection of the BAC DNA partially rescued slj exocrine pancreas defects (Figure 2B; n = 6 of 17 injected slj larvae), thus confirming that the BAC spanned the slj locus.

Sequence analysis from the zebrafish genome project identified three genes within BAC zk103i16 adjacent to the slj locus (Figure 2A). Further meiotic mapping narrowed the critical interval to a region that contained the polr3b gene (http://www.sanger.ac.uk/Projects/D__rerio). We then scanned polr3b cDNA for mutations. Reverse-transcriptase PCR (RT-PCR) products and their sequencing identified a 123-bp deletion in polr3b cDNA amplified from slj mutant larvae, but not from homozygous wild-type larvae (Figure 2C and 2D). Additional analyses indicated that the 123-bp deletion corresponded precisely to exon 10 of the polr3b gene [27]. A smaller deletion, comprising 66 nucleotides from exon 10, was subsequently identified in a minority (~10%) of the polr3b cDNA fragments (Figure 2E and 2F). Both deletions are predicted to occur in-frame, thus generating polr3b cDNAs
acceptor of the cytosine transition was present in the intron 10 splice donor. Sequences analyses confirmed this prediction: a thymine-to-cytosine transition caused the exon 10 skipping induced by the slj mutation. Sequence analyses confirmed this prediction: a thymine-to-cytosine transition was present in the intron 10 splice donor. RT-PCR and sequencing analyses shown in Figure 2E and 2F were performed on whole embryos, the data indicate that the slj mutation leads to altered splicing with in frame codon deletions in the vast majority, if not all, of the polr3b transcripts.

To confirm that the polr3b mutation was responsible for the slj phenotype we designed antisense Morpholinos that targeted the polr3b mRNA (Figure 3). Microinjection of a Morpholino targeting the translation initiation codon (5′-ATG) led to early lethality (prior to 24 hpf) in the majority of injected embryos, with the remainder showing severe developmental delays (unpublished data). Injection of a lower dose of this Morpholino produced an slj intestinal and pancreatic phenocopy in approximately 50% and 60% of surviving wild-type 5-dpf larvae (n = 77 and 47 embryos analyzed, respectively, in two independent experiments; Figure 3A–3C and 3E–3G). We also designed a Morpholino spanning the intron 9 splice donor with the hope that targeting this site would induce deletion of exon 10. Indeed, RT-PCR and DNA sequence analysis of the polr3b cDNA derived from embryos microinjected with this Morpholino, but not control-injected embryos, revealed in-frame deletion of exon 10 of the polr3b cDNA (Figure 3I). Importantly, 52% and 43% of surviving embryos injected in two independent experiments showed an slj intestinal and pancreatic phenocopy (n = 33 and 53 total embryos analyzed, respectively; Figure 3D and 3H).

In summary, mutant phenocopy by two nonoverlapping Morpholinos confirm identification of polr3b as the gene targeted by the slj mutation.

polr3b Expression during Zebrafish Development

To define the location and levels of polr3b expression in developing zebrafish embryos and larvae, we performed RNA whole-mount in situ hybridization assays and quantitative real-time RT-PCR amplification of the polr3b cDNA (Figure 4). These data showed strong maternal polr3b expression (Figure 4A) and strong zygotic polr3b expression that peaked at 24 hpf and subsequently declined (Figure 4B–4F). Beyond 3 dpf, we observed only low levels of polr3b expression in the digestive organs, that were only slightly above background and thus difficult to image (unpublished data). This decline in polr3b expression coincides with 5-fold reduced cell proliferation in the digestive system and other tissues between 2 dpf and 4 dpf [22]. This relatively low level of Polr3b expression encoding proteins truncated by internal deletion of either 22 or 41 amino acids.

Exon 10 skipping induced by the slj mutation could arise from disruption of the polr3b intron 10 splice acceptor. Sequence analyses confirmed this prediction: a thymine-to-cytosine transition was present in the intron 10 splice acceptor of the polr3b 3′UTR, but not the wild-type polr3b allele (Figure 2G). This single intronic substitution causes the exon

Table 1. Cell Proliferation Indices in Wild-Type and slj Larvae

| Cell Type | Age (hpf) | % BrdU Cells | % PH3 Cells |
|-----------|-----------|--------------|-------------|
|           |           | wt slj p-Value | wt slj p-Value |
| InE       | 60        | 45.5 (8.8) 39.6 (2.9) 0.33 | 2.0 (1.5) 1.3 (1.5) 0.53 |
|           | 72        | 35.4 (6.4) 19.8 (5.3) 0.03 | 1.8 (1.6) 1.3 (0.5) 0.26 |
| InS       | 60        | 17.7 (0.1) 22.1 (1.7) 0.01 | NC NC — |
|           | 72        | 10.2 (3.5) 9.3 (0.7) 0.68 | NC NC — |
| ExP       | 60        | 63.4 (5.6) 59.1 (15.7) 0.68 | 2.6 (1.9) 1.1 (1.7) 0.33 |
|           | 72        | 50.9 (2.3) 10.7 (2.1) <0.001 | 1.9 (1.1) 1.3 (0.9) 0.44 |

ExP, exocrine pancreas; InE, intestinal epithelium; InS, intestinal stromal cells; NC, not counted; wt, wild type.
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Figure 1. The slj Mutation Disrupts Intestinal and Exocrine Pancreas Development

(A–D) Lateral views of 5-dpf wild-type (A) and slj (B) larvae, with representative histological cross-sections (C and D). The slj intestine is small, thin walled, and lacks folds (arrows, [A and B]) compared with wild type, and the slj terminal branchial arches are reduced (arrowheads). The columnar morphology and apical microvilli of the slj epithelium are less developed than in wild-type larvae (C and D); an asterisk (*) indicates the intestinal lumen.

(E and F) The size of the slj liver (indicated by red asterisk in [A] and [B]) and the retinae (arrows, [E and F], dorsal view) are also reduced. (G and H) Although exocrine tissue is well developed in wild-type embryos [22], it is markedly reduced or absent in slj embryos (H); asterisks indicate intestine. By contrast, the slj and wild-type pancreatic islets are of comparable size (arrows).
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Pol3 Mutation Disrupts Organ Growth

To determine the location and levels of polr3b expression in developing zebrafish embryos and larvae, we performed RNA whole-mount in situ hybridization assays and quantitative real-time RT-PCR amplification of the polr3b cDNA (Figure 4). These data showed strong maternal polr3b expression (Figure 4A) and strong zygotic polr3b expression that peaked at 24 hpf and subsequently declined (Figure 4B–4F). Beyond 3 dpf, we observed only low levels of polr3b expression in the digestive organs, that were only slightly above background and thus difficult to image (unpublished data). This decline in polr3b expression coincides with 5-fold reduced cell proliferation in the digestive system and other tissues between 2 dpf and 4 dpf [22]. This relatively low level of Polr3b expression...
Figure 2. The slj Locus Encodes Zebrafish polr3b, the Gene Encoding the Second Largest Subunit of RNA Pol III
(A) Schematic of the region surrounding the slj locus. Genetic markers and the number of recombinants are listed above the names of the genes and expressed sequence tags (ESTs) that map to this region of zebrafish Chromosome 18. A zero recombinant marker (G)14, is located within the polr3b coding region. Arrows denote orientation of gene transcription. Red asterisk (*) denotes location of slj mutation.
(B) High-power, lateral views of the immunostained pancreas from a 5-dpf wild-type zebrafish larva (left panel), and two slj larvae microinjected with either phenol red vehicle (middle) or the BAC spanning the slj locus (right panel). All larvae were processed for insulin (ins), red) and carboxypeptidase A (cpa, green) immunohistochemistry. A normal pattern of immunoreactive insulin is present in all larvae. BAC injection restores cpa staining and therefore partially rescues the cpa-positive exocrine pancreas cells in the BAC-injected larva and note their absence in the slj larva.
(C) Predicted amino acid sequence encoded by the human (Hs) and zebrafish (Dr) Polr3b cDNA surrounding the region deleted by the slj mutation. Two polr3b mRNA splice variants are generated by the slj mutation: the slj-b variant lacks all 41 amino acids encoded by exon 10 (aa239–279) of the polr3b gene, whereas the slj-a variant lacks 22 exon 10 amino acids (aa258–279). (D) PCR amplification of cDNA derived from pooled 5-dpf slj larvae and homozygous wild-type (WT) siblings using primers that span polr3b exon 10, as depicted in (E). Sequencing revealed that a 278-bp band is amplified from wild-type larvae, whereas a 155-bp band corresponding to deletion of exon 10 is amplified from slj larvae.
(E and F) Schematic (E) depicting the wild-type and slj polr3b mRNAs exons 9–11 (E9, E10, and E11). Arrows refer to PCR primers used to quantify the relative proportion of the slj-A and slj-B transcripts (F) in the 5-dpf slj and wild-type larva shown in (D).
(G) Schematic depiction of the DNA sequences encoded by wild-type and slj polr3b alleles. The slj mutation encodes a thymine (T) to cytosine (C) transition in the intron 10 splice acceptor.

may sensitize cells to the slj mutation, and provide an explanation as to why the digestive system and other proliferative tissue may be selectively affected in slj mutants (see Discussion).

To determine whether the slj mutation disrupted Pol III function, we quantified the levels of Pol III target gene RNAs in wild-type and slj larvae. We used an Agilent Bioanalyzer for high-resolution analysis of total RNA (see Materials and Methods). This revealed decreased levels of total tRNAs in 4-dpf and 5-dpf slj larvae, but normal 5S and 5.8S rRNA levels (Figure 5A). Normal 5.8S RNA levels in slj larvae (unpublished data) were expected because this gene is transcribed by Pol I. Discordant effects of the slj mutation on 5S rRNA and tRNA gene expression, which are both transcribed by Pol III, were also not surprising, given similar findings with mutation of yeast Pol III subunit genes [28]. Such differences in the sensitivity of Pol III target genes has been attributed to variable specificity of the Pol III complex for its target gene promoters [28]. Such specificity may also explain the variable effects of the slj Pol III defect on tRNA levels (discussed below). However, it is also possible that reduced 5S rRNA transcription and transcription of specific tRNAs in slj larvae is compensated by enhanced transcript stability.

To confirm these data, we assessed the effect of the slj mutation on individual Pol III target genes. Quantitative RT-PCR experiments showed that there were reduced levels of a tRNA-leu in 3-dpf and 5-dpf slj larvae relative to the levels of the Pol II target gene hprt (Figure 5B). Interestingly, tRNA-leu levels were normal in 4-dpf slj larvae, thus revealing a complex relationship between Pol III activity and target gene transcription. Expression of the 7SL RNA, a component of the signal recognition particle that is also transcribed by Pol III [29], was also reduced in 4-dpf and 5-dpf slj larvae (Figure 5C). Reduced levels of mature tRNA-leu and a tRNA-ile were also confirmed by northern analyses of 5-dpf slj larvae. Levels of these tRNAs were 48% and 36%, respectively, of their sibling wild-type larvae relative to the expression of the Pol II transcript U1 snRNA on the same blot (Figure 5D). Reduced levels of mature tRNAs suggest reduced efficiency of the slj Pol III enzyme, but do not exclude post-transcriptional effects [25,30,31]. Because of the rapid rate at which pre-tRNAs are processed, they are considered to be reliable indicators of Pol III transcription rate [16,32]. Therefore, we used northern blot analysis and quantitative PCR to measure pre-tRNA levels in slj and sibling wild-type larvae, relative to the Pol II transcribed genes U1 snRNA and hprt mRNA (Figure 5E and 5F, respectively). The levels of both of the pre-tRNAs examined were reduced in 5-dpf slj larvae compared with wild-type siblings, supporting the idea that Pol III transcription is reduced in slj mutants.

Cross-Species Analyses Suggest That the slj Mutation Disrupts Interaction of Polr3b (Rpc2) and Polr3k (Rcp11) Subunits
Although high-resolution analysis of the 17 subunit Pol III structure has not been defined, a homology-based model of the nine-subunit core yeast Pol III complex and the structure of an associated subcomplex have been reported [3]. In addition, the 17-subunit complex has been visualized, with some of its subcomplexes localized by electron microscopy [4]. The Pol III structure derived from these studies and the original studies describing the Pol II structure [33,34] suggest a high degree of homology, especially in the largest subunits and their contacts with other subunits (most of which are either shared by Pol II and III, or highly homologous [3,4]). Since Rpc2p is highly homologous to the second largest Pol II subunit, Rpb2p, the Pol II structure provided a guide to how the slj deletion might affect Pol III function. Examination of the yeast Pol II structure revealed that the region of subunit Rpb2p corresponding to the Rpc2 slj deletion makes contact with Rpb9p (discussed in [4]), a subunit homologous to the Pol III subunit, Rcp11p [24]. This suggested the possibility that the region deleted in slj Polr3b might contact the
zebrafish Rpl11p ortholog, Polr3k. This idea is supported by two hybrid studies of yeast Pol III that show interaction between Rpl11p and the N-terminal region of Rps2p [5].

To further explore whether the slj Polr3b deletion might compromise interaction with Polr3k, we engineered S. pombe to express a mutant Rps2p with a deletion (Rps2Δp) corresponding to the evolutionarily conserved region deleted by the polr3b slj mutation, and examined its subunit interactions. Hemagglutinin (HA)-tagged versions of wild-type and mutant S. pombe rps2 were introduced into an S. pombe strain containing a His6-FLAG-tagged Rps3p subunit, and Pol III complexes containing the tagged subunits were recovered by sequential affinity immunoprecipitation as described in Materials and Methods. Western blot analysis of multiple subunits revealed a markedly reduced amount of Rps11p in the Pol III complex containing Rps2Δp (Figure 6A, lane 8) relative to wild-type Rps2p (Figure 6A, lane 7). This experiment supports the idea that exon 10 of zebrafish polr3b encodes amino acids crucial for the stable interaction of the zebrafish orthologs of Polr3b and Polr3k (Rps2 and Rps11), and suggests that the slj phenotype might be due, at least in part, to instability or failure of this interaction in vivo.

Overexpression of Zebrafish polr3k Rescues slj Exocrine Pancreas Defects

Pol III subunit interactions in yeast have largely been defined via two-hybrid analyses or the more functional approach, overexpression–suppression experiments. The latter approach involves suppression of hypomorphic phenotypes by overexpression of a gene encoding an interacting subunit. To adapt this approach to the possibility that the slj mutation might affect the efficiency of the Polr3b–Polr3k interaction, we injected zebrafish polr3k cDNA driven by the heat-inducible hsp70 promoter, which drives high levels of gene expression throughout the embryo (albeit in a mosaic fashion; [35]) in transient expression assays. This led to partial but highly significant rescue of the slj exocrine pancreas defect (Figure 6B; n = 8 of 13 and n = 9 of 9 injected slj larvae rescued from two independent experiments). This rescue, which in most larvae was more pronounced than that achieved with the BAC injection (Figure 2B), is consistent with data on Rps11p in yeast two-hybrid analyses of Rps2p-Rps11p, and the predicted interaction between these subunits in the structures noted above. Suppression of the slj phenotype by microinjection-overexpression of Polr3k provides strong evidence to indicate that the slj phenotype is due, in significant part, to the deficiency of a stable interaction between Polr3k (Rps11) and Polr3b (Rps2) in vivo.

Polr3k-mediated rescue of the slj exocrine pancreas defects suggests that a decrease in the concentration of Polr3k may produce a phenocopy of the slj phenotype. To explore this hypothesis, we again used antisense Morpholino-mediated knockdown in developing zebrafish, this time targeting Polr3k. Consistent with this idea, injection of a Morpholino targeting the translation initiation codon (5′-ATG) of the
Discussion

The major conclusion that can be drawn from this study is that a mutation in the zebrafish second largest Pol III subunit (Polr3b), that impairs its association with another Pol III subunit (Polr3k), causes tissue-specific defects in zebrafish development. These defects appear to result from failure of adequate proliferation and growth of cells that have a subcritical level of active Pol III. Strikingly, much of the developmental defect in the exocrine pancreas can be overcome in the Polr3b mutant fish by overexpression of Polr3k. These data indicate the importance of the Polr3b–Polr3k interaction in Pol III function, as well as the effect of reduced Pol III activity in tissue-specific development.

Because zebrafish slj larvae contain less tRNA and 7SL RNA, as well as other Pol III transcripts, it is presumed that the developmental defects observed in slj larvae result from a deficiency of small RNAs that regulate essential cellular processes such as ribosome biogenesis, protein synthesis, and cell-cycle progression [1,6]. This is particularly important to dividing cells, as evidenced by numerous studies showing the effects of reduced translational output on cell growth and cell-cycle progression (discussed in [36]). For example, a 2-fold reduction in tRNAiMet levels is associated with a 3-fold decrease in the doubling time of yeast [37]. Disruption of Pol III transcription can also activate stress-response pathways and indirectly disrupt cross-talk between Pol III and the Pol II transcriptome [38]. Such an effect may also contribute to the developmental defects imparted by the slj mutation.

Given the importance of Pol III, it is not surprising that cells contain a number of mechanisms to regulate its activity. Recent studies in mammalian cells have focused on how tumor suppressors, oncogenes, and cell-cycle proteins can either restrict or enhance recruitment of Pol III to its target gene promoters [8–14]. This control may involve up-regulation of the genes encoding Pol III transcription factors, altered protein–protein interactions between the transcription factors, or alternatively, a direct effect on Pol III itself [13,14]. The results reported here are distinguishable from these studies because they suggest that tissue-specific developmental defects in highly proliferative tissues may arise from impaired interaction between two Pol III subunits.

Although deregulated Pol III activity is a common feature of tumorigenic and hypertrophic cell growth in cultured cells [13,14,39], evidence that elevated Pol III activity is required for phenotypic transformation in a multicellular organism has been lacking. Thus, our finding that reduced Pol III transcription in zebrafish slj mutants disrupts growth and development that is dependent on cell proliferation in the digestive system, retinae, and other highly proliferative larval tissues is noteworthy. Similarly, whereas there was considerable data showing that reduced tRNA levels appear to limit the proliferation of cultured cells, the present report illustrates the potential importance of this mechanism in a multicellular organism. We found that the Pol III defect imparted by the slj mutation had a far more pronounced effect on actively cycling cells than on tissues principally populated by quiescent postmitotic cells, such as skeletal muscle, heart, and the pancreatic islet. These data further support the idea that regulation of Pol III activity is integral to cell growth and proliferation in vertebrate tissues, and moreover, illustrate tissue-specific developmental defects that arise as a result of not meeting the Pol III–dependent demands of such cells. Finally, although it is possible that the development and function of quiescent cell types in slj mutants may be sustained by maternally derived Polr3b protein, the metabolic demand of developing cells may exceed maternal supply (as described for genes encoding subunits of the coatomer complex in the developing zebrafish notochord [40]). Thus, the sensitivity of tissue progenitor cells, rather than quiescent cells, to reduced Pol III activity
caused by polr3b mutation may reflect such metabolic demands.

Although we have not compared Pol III activity levels in the tissues that were differentially affected by the slj mutation, our data suggest that the threshold of Pol III activity required to sustain development of different cell types within a specific tissue is variable. We found that intestinal stromal cell proliferation was increased in early slj larvae (InS, 48 hpf; Table 1), but was normal at a slightly later stage, when epithelial proliferation is significantly reduced (72 hpf). We also found that the effect of the slj mutation varied between different tissues, with a far more pronounced effect on exocrine pancreas development than intestinal development [21–23]. A second possibility that is consistent with our data is that developing cells might require different levels of Pol III activity for their differentiation programs. This is consistent with the finding that although there is normal differentiation of most epithelial lineages in the slj intestine, epithelial morphogenesis is abnormal [22].

A third idea to emerge from this work is that vertebrate cells may regulate Pol III activity, in part by regulating its subunit gene expression. Even when the overall expression level of polr3b is low, such as on 3 dpf and at later stages, it was consistently most pronounced in the tissues that have a high percentage of proliferative cells, such as the intestine and pancreas (Figure 4). Consistent with this idea, it was previously shown that expression of the gene encoding the Pol III subunit Rcp53/Rpol3d (also known as BN51) increases in response to serum stimulation of cell proliferation [41].

Conserved Pol III Subunit Interactions in Yeast and Zebrafish

Mutants targeting yeast Rcp2p had been isolated by a genetic screen that selected for impaired termination by Pol III, and were later found to affect elongation rate with reciprocal effects on termination [42–45]. Because the slj mutation was adjacent to some of these yeast rcp2 mutations, and because rcp11 was linked to Pol III termination and RNA 3’-end formation [24,25], we wanted to know whether termination was affected in slj mutants. A minimal efficiency vertebrate Pol III terminator consists of a run of four thymidine (T) residues, with termination efficiency increasing as the number of T residues increases [46]. Transcription beyond a weak Pol III terminator in vivo can be visualized on northern blots as pre-tRNA transcripts that extend beyond the 3’ terminator into flanking DNA [47]. To examine this possibility, we identified a tRNAxile gene with a minimal 4T terminator and examined its transcripts in wild-type and slj mutant fish. Using an intron probe as well as a 3’ flanking probe designed to detect read-through transcripts that extend beyond the terminator, we found no evidence of a difference in the Pol III termination efficiency of this gene in wild-type and slj larvae, since the pre-tRNA-Ile transcript size was identical in slj and wild-type larvae, with no evidence of longer transcripts on the blot (Figure 5E and unpublished data). These data are consistent with the finding that function-altering mutations in rcp11 did not affect termination efficiency in fission yeast [25] and a revised role for Rcp1lp in Pol III recycling rather than termination per se [26]. Recycling, or facilitated reinitiation, is a feature of the high efficiency of Pol III [26]. Given evidence of no impairment of termination by slj Pol III, we speculate that reduced reinitiation may be the cause of decreased Pol III transcription in slj mutants.

Contrary to what might be expected from the study of a Saccharomyces cerevisiae rcp11 mutant that contained wild-type S. pombe Rcp2p [24], Pol III purification from S. pombe revealed similar Rcp53p levels in the wild-type and slj Rcp2-Ap cells (Figure 6A). This may be explained by the fact that the genetic approaches differed (small deletion in Rpc2 in S. pombe, versus replacement of S. cerevisiae Rpc11 with S. pombeRpc11 in S. cerevisiae) and that very different Pol III purification schemes were used (epitope tag affinity chromatography versus extensive ion exchange chromatography) in the present and prior studies, respectively.

In summary, the work described in this study reveals an unexpected importance of the Rcp2–Rcp11 interaction during development and demonstrates the utility of the zebrafish system. We have shown that in vivo analyses of Pol III function are feasible in zebrafish and that they complement analyses in other model systems. We have also shown that the zebrafish may be used to reveal the effects of disrupting Pol III in complex multicellular tissues. An idea suggested by these studies is that specific cell types within a
multicellular organism may require different levels of Pol III activity and that this may reflect their rate of proliferation and/or growth. Thus, it is conceivable that selective Pol III inhibitors may be able to target metabolically active cells in proliferative or hypertrophic disease states.

Materials and Methods

Positional cloning of slj locus. The slj mutation bulk-segregant analyses identified two Chromosome 18 markers linked to the slj locus (M. Mohideen, M. Fishman, and M. Pack; unpublished data). Subsequent analyses identified two closely linked markers, z15417 and z21530, that were 0.08 cM and 0.92 cM from the slj mutation (three and five recombinants from 3,612 meioses and one recombinant from 4,186 meioses). A BAC clone zK130H16 spanning the critical region bounded by these markers was identified. Within this BAC, two additional polymorphic markers, CA11 and GT14, were identified. Meiotic mapping showed that one and zero of 1,806 slj larvae were recombinant for these markers. Sequence analyses indicated that the two markers, CA11 and GT14, were located within intron 23 and intron 10 of the polr3b gene, respectively.

BAC rescue experiments. To verify that BAC clone zK130H16 spanned the slj locus, phenotype rescue experiments were performed. BAC rescue BACs containing a coding region were amplified using a commercial BAC DNA kit (Princeton Separations). The BAC DNA (1 nl of 12.5 ng/µl in 0.1% phenol red) was microinjected into the progeny of a slj-/-intercross at the one-cell embryonic stage. Equivalent numbers of embryos were injected with phenol red solution as control. The embryos were raised to 4 dpf and assayed by anti-carboxypeptidase A and anti-insulin immunohistochemistry as described [21-25].

Morpholino injection. Morpholinos targeting the polr3b and polr3k mRNAs were injected into newly fertilized one-cell to four-cell stage zebrafish embryos as previously described [48]. The sequences of the Morpholinos are:

**polr3b** ATG_CAGGAGATTTCACCACTGATAG
control: TTCAACTTCTCTTGTCTCAGTGC

**polr3k** ATG_TTTCCCCGGACTCCTCTTGCAGCATA
splice: TTTCCCCGGACTCCTGTCCTCAGAAG

**polr3k rescue experiments.** For the polr3k expression construct, PC primer designs were that were complementary to the 5’ and 3’ regions of the zebrafish polr3k ortholog identified in the zebrafish database. The forward and reverse primer sequences are GA-TATGCTGTGAAATGCTCTGGTTTACT and ACTAGTAAAGTGAATATCATCTGCTACACTG. The resulting PCR fragment was 2.6 kb. To replace a FLAG tag with a HA tag in Rcp2p and Rcp2p-Δp, PCR was performed with primers retremfor3 (5’-CATGTCGACATGGAT-3’) and retremrev3 (5’-CATGTCGACATGGAT-3’), in which rpc53 is tagged upstream of the coding region of RCP2, was deleted by PCR using primers 5’-CACTATAGGAATATCATCTGCTACACTG). The resulting RPC2 was transformed into YH3262 [50] using pREP5X, producing pREP5X-HA-rpc2p and pREP5X-HA-rp2-Δp, respectively. To purify wild-type Pol III and Pol III mutants containing Rcp2p-Δp, prpE5X-HA-rp2pc and prpE5X-HA-rp2-Δp were transformed into yH3262(+), his3-D1, leu1-32, ura4-D1, met15-216, in which rpc53 is tagged with FLAG and six histidine residues. Purification of wild-type and mutant Pol III was carried out as previously described [50] with the following modifications: the eluate from the Ni-NTA column was incubated with 20 µl of anti-HA beads for 4 h at 4°C, the bound proteins were eluted by boiling the beads in 2×tris-glycine SDS gel loading buffer.

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the proteins discussed in the paper are as follows: human Polr3b (NM_018082) and mouse Polr3b (NM_027423).

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