Zebrafish usp39 Mutation Leads to rb1 mRNA Splicing Defect and Pituitary Lineage Expansion

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Abstract

Loss of retinoblastoma (Rb) tumor suppressor function is associated with human malignancies. Molecular and genetic mechanisms responsible for tumorigenic Rb downregulation are not fully defined. Through a forward genetic screen and positional cloning, we identified and characterized a zebrafish ubiquitin specific peptidase 39 (usp39) mutation, the yeast and human homolog of which encodes a component of RNA splicing machinery. Zebrafish usp39 mutants exhibit microcephaly and adrenocorticotrophic cell lineage expansion without apparent changes in major hypothalamic hormonal and regulatory signals. Gene expression profiling of usp39 mutants revealed decreased rb1 and increased e2f4, rb2 (p130), and cdkn1a (p21) expression. Rb1 mRNA overexpression, or antisense morpholino knockdown of e2f4, partially reversed embryonic pituitary expansion in usp39 mutants. Analysis of pre-mRNA splicing status of critical cell cycle regulators showed misspliced Rb1 pre-mRNA resulting in a premature stop codon. These studies unravel a novel mechanism for rb1 regulation by a neuronal mRNA splicing factor, usp39. Zebrafish usp39 regulates embryonic pituitary homeostasis by targeting rb1 and e2f4 expression, respectively, contributing to increased adrenocorticotrophin sensitivity to these altered cell cycle regulators. These results provide a mechanism for dysregulated rb1 and e2f4 pathways that may result in pituitary tumorigenesis.

Introduction

The hypothalamic-pituitary axis regulates stress responses, growth, reproduction and energy homeostasis. Neuropeptides released from the hypothalamus via the hypophyseal portal plexus control synthesis and secretion of anterior pituitary hormones [1]. Different pituitary cell types secrete hormones that regulate post-natal growth (growth hormone, GH), lactation (prolactin, PRL), metabolism (thyroid stimulating hormone, TSH), stress (adrenocorticotrophic hormone, ACTH), pigmentation (melanocyte-stimulating hormone, α-MSH, and follicle stimulating hormone, FSH) [2]. Corticotropes and melanotropes produce proopiomelanocortin (POMC), which is proteolytically cleaved to give rise to ACTH in corticotropes and α-MSH in melanotropes.

Central and peripheral signals including hypothalamic stimulatory hormones, growth factors and estrogen cause pituitary hyperplasia, genetic instability, subsequent monoclonal growth expansion and tumor formation [3]. Pituitary tumors are almost invariably benign, however if untreated, they are associated with increased morbidity and mortality due to tumor mass effect and/or hormonal disruptions leading to serious complications such as acromegaly and Cushings’ disease [4,5]. How developmental or acquired signals elicit plastic change in pituitary cell growth resulting in hyperplasia or benign adenomas is not fully understood [6].

The pituitary gland is highly sensitive to cell cycle regulators including cyclins, cyclin dependent kinases (CDKs), CDK inhibitors (CKIs) and retinoblastoma protein (pRB), all of which are frequently dysregulated in pituitary tumors. pRB, a nuclear pocket protein, binds the E2F transcription factors and regulates the balance between cell quiescence and proliferation [7]. E2Fs control expression of genes crucial for cell cycle re-entry, DNA replication and mitosis. Dephosphorylated pRB binds to E2Fs and inhibits transcription of E2F target genes either by sequestration and inhibition of E2F cell cycle “activators” (E2F1–E2F3), or by formation of pocket protein complexes with “inhibitors” (E2F4–E2F8), which bind to E2F-responsive promoters and repress their transcription [7]. Accordingly, transcriptional repression of pRB activity prevents G1/S progression and promotes cell quiescence.

In mice, Rb heterozygous mutations lead to early onset and increased incidence of endocrine neoplasma including pituitary, thyroid and adrenal tumors [8,9]. The 100% penetrance of pituitary tumors in Rb+/− mice [8] is partially reversed in Rb+/−; E2f4−/− double mutants, implicating the Rb/E2f4 pathway in pituitary tumorigenesis and also suggesting an E2F4 oncogenic activity [9]. E2f4 is also known as a key regulator associated with p130 in G0/G1 to promote quiescent G0 and terminal differentiation [10,11]. E2f4 null mice often die shortly after birth with defects of terminal differentiation resulting from an inability to establish cell cycle quiescence [12]. In response to cell cycle re-entry, E2f4 switches from p130 [10,13] to pRB [10,14] and p107 [10,14,15], which inhibit E2f4 transactivation. Additionally, E2f4 overexpression has been shown to promote cell proliferation and transformation [14,15], which prevents growth arrest mediated by p130 [13].
Pituitary development and physiology are conserved in zebrafish [2]. Novel insights into developmental mechanisms have been obtained by in vivo analysis of transgenic zebrafish expressing GFP and RFP driven by regulatory elements of zebrafish pone [16] and prf [17], respectively. Through a forward genetic screen for novel zebrafish genes regulating adenohypophyseal pone gene expression, we identified and characterized a mutant that harbors a nonsense mutation in usp39, leading to expansion of all adenohypophyseal cell lineages. 

Usp39 encodes a conserved protein termed Sad1p in Saccharomyces cerevisiae and a 65 kDa (65k) SR-related protein in humans [18,19]. Both yeast Sad1p and the 65k SR-related protein in humans are involved in assembly of the spliceosome, the RNA splicing machinery [18,19]. RNA splicing is crucial for eukaryotic gene expression and defective splicing can be detrimental since it leads to an altered genetic message [20]. The spliceosome consists of five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5 and U6 as well as a large number of non-snRNP proteins [20]. The yeast Sad1p is involved in splicing in vivo and in vitro and in the assembly of U4 snRNP to U6 snRNP [18], while human 65k SR-related protein is essential for recruitment of the tri-snRNP to the pre-spliceosome and is known as a tri-snRNP-specific protein [19,21]. Additionally, Usp39 is also classified as a deubiquitinating enzyme but lacks protease activity due to the absence of key active-site residues of cysteine and histidine [18,19,22]. 

In the present study, we aimed to define novel pathways regulating pituitary development through study of an usp39 mutation. Using microarray gene expression profiling followed by quantitative real time-polymerase chain reaction (RT-PCR) validation we observed a significant reduction of rh1 expression and increased e2f4, rh2 (p130) and cdkn1a (p21) expression in mutants. Zebrafish usp39 is predominantly expressed in the brain and represents a novel neuronal splicing factor. We show that zebrafish usp39 mutation leads to an rh1 splicing defect responsible for pituitary expansion. In addition, knockdown of e2f4 partially rescued pone lineage expansion in usp39 mutants. Our finding that usp39 regulates expansion of all embryonic pituitary cell lineages through the rh1/e2f4 pathway may shed light on mechanisms underlying adult pituitary tumor formation.

Results

The hp689 locus encodes usp39

To isolate genes required for adenohypophysis and hypothalamic development a standard forward genetics method was carried out using a three-generation (F3) screen after mutagenesis with ENU, which mostly induces single nucleotide exchanges at random positions of the genome [23–25]. The genetic screen was performed using pone expression as a specific marker. pone is expressed in subependymal pituitary cells, dorsal to the oral ecdoterm roof and ventral to the ventre diencephalon. A subset of pone-expressing cells is also located outside the adenohypophysis, corresponding to \( \beta \)-endorphin-synthesizing cells of the hypothalamic arcuate nucleus [2]. In zebrafish, spatial distribution of the six different hormone secreting pituitary cell types is subdivided into three regions along the antero-posterior adenohypophyseal axis of the rostral pars distalis, proximal pars distalis and pars intermedia [2]. pone is expressed in corticotropes of the rostral pars distalis, in melanotropes of the pars intermedia and in the hypothalamus (Figure 1A).

We isolated an ENU-induced mutant, hp689, which was characterized by reduced hypothalamic but increased pituitary pone expression at 40-hours post fertilization (hpf) (Figure 1B). In addition, hp689 mutants displayed microcephaly and smaller eyes starting at 33 hpf (data not shown). Using segregation linkage analysis, the hp689 locus was mapped to zebrafish linkage group 5 with a critical interval of 0.03 centimorgan (cM) on marker ndrg3 (Figure 1C, see Materials and Methods). This region contained 7 annotated genes and sequencing of usp39 from mutant embryos revealed a point mutation that converted a TAT codon to a TAA in exon 11, resulting in a premature termination codon rather than a tyrosine amino acid (Figure 1D). PROSITE database search of the Usp39 protein revealed two domains consisting of a zinc finger (ZF_UBP) and a Ubiquitin carboxyl-terminal hydro-lases family 2 (UCH_2_3) region [26]. As a result of the UCH_2_3 domain, Usp39 is classified as a deubiquitinating enzyme. However, it lacks protease activity due to the absence of key active site cysteine and histidine residues [18,19,22]. The single allele of hp689 carries a nonsense mutation within the UCH_2_3 region, resulting in a truncated Usp39 protein lacking amino acids after position 412 (Figure 1D).

To confirm that hp689 represents the usp39 mutation, a usp39 antisense morpholino (MO) oligonucleotide targeting the usp39 start codon and consequentially blocking translation was injected into wild-type (wt) embryos [27]. MO injected embryos showed increased pone expression, similar to the hp689 phenotypic Figure 1F compared to Figure 1B). Furthermore, injection of usp39 RNA encoding wild-type usp39 rescued the mutant phenotype (Table 1), indicating that pituitary pone upregulation in usp39 mutants results from the nonsense mutation in zebrafish usp39.

usp39 is predominantly expressed in the zebrafish embryonic brain

RNA whole-mount in situ hybridization was performed to determine the spatiotemporal expression pattern of usp39 during zebrafish development. Generally weak usp39 expression was detected in early cleavage embryos (data not shown) but tissue specific expression peaked at 36 hpf and decreased by 42 hpf (Figure 2A–2C). Expression was detected predominantly within the brain, including the pituitary region and eyes. At 21.5 hpf, there was also expression in the intermediate cell mass, the site of embryonic zebrafish hematopoiesis (Figure 2A, inset). However, mutants showed persistently lower usp39 expression that was completely lost by 42 hpf (Figure 2D). In addition, loss of usp39 expression by 42 hpf corresponds to the time at which the usp39 mutant embryos fully develop a phenotype of microcephaly, smaller eyes and a pituitary abnormality, indicating the critical time point when usp39 is required for normal development.

Author Summary

Previous studies have shown that \( \beta \)-hormone−/− mice develop pituitary adenomas; however, \( \beta \)1 mutations have not been found in human pituitary tumors. In the present study, we uncovered a novel genetic pathway that may lead to \( \beta \) downregulation through RNA splicing mediated by usp39, a gene involved in assembly of the spliceosome. Our forward genetic study in zebrafish suggests that loss of usp39 results in aberrant \( \beta \) mRNA splicing, which likely causes elevated expression of its target e2f4, a key regulator known to have oncogenic activity when overexpressed. We established that e2f4 upregulation is a main factor responsible for the adenohypophyseal cell lineage hyperplasia observed in the zebrafish usp39 mutant. It should be of interest to investigate if mutations or downregulation of USP39 would contribute to pituitary tumorigenesis in humans.
The zebrafish adenohypophysis consists of six different hormone-secreting cell types distributed along the anterior-posterior axis: lactotropes and corticotropes are located anteriorly in the rostral pars distalis, thyrotropes, gonadotropes and somatotropes are found medially in the proximal pars distalis whereas melanotropes are situated posteriorly in the pars intermedia (Figure 3). To determine if additional pituitary lineages are affected by the usp39 mutation, we performed double color RNA in situ hybridization analysis with combinatory pituitary markers. Table 1. usp39 mRNA overexpression rescues the mutant usp39 phenotype.

Pituitary cell lineage expansion in usp39 mutants

The zebrafish adenohypophysis consists of six different hormone-secreting cell types distributed along the anterior-posterior axis: lactotropes and corticotropes are located anteriorly in the rostral pars distalis, thyrotropes, gonadotropes and somatotropes are found medially in the proximal pars distalis whereas melanotropes are situated posteriorly in the pars intermedia (Figure 3). To determine if additional pituitary lineages are affected by the usp39 mutation, we performed double color RNA in situ hybridization analysis with combinatory pituitary markers.
lineage specific marker genes pome, gh, prl, tsh, and with cga that encodes the glycoprotein α-subunit heterodimerizing with TSHβ, LHβ, or FSHβ subunit [2,16]. This analysis revealed expansion of all the analyzed cell lineages without apparent cell fate transformation in the usp39 mutant pituitary at 48 hpf (Figure 3A–3P). Cell expansion was most marked in corticotropes and lactotropes, indicating that usp39 is important for regulating embryonic pituitary cell populations (Figure 3B, 3D, 3F, 3H, 3J, and 3L).

Pituitary lineage expansion in usp39 mutants is independent of hypothalamic releasing hormone and dopamine signals

We examined expression of hypothalamic regulators to investigate whether pituitary lineage expansion in usp39 mutants is due to altered hypothalamic neuroendocrine input to the adenohypophysis. One of the primary hypothalamic inhibitory mechanisms controlling pituitary homeostasis is dopamine (DA) released from tuberoinfundibular neurons (TIDA). Pituitary lactotrophs are almost exclusively regulated by tonic inhibition of dopamine, which inhibits lactotroph proliferation, PRL gene expression and secretion by activating D2 dopamine receptor subtype (Drd2) [28]. We therefore processed 48 hpf whole-mount embryos for immunocytochemistry using an antibody against tyrosine-hydroxylase, the rate-limiting enzyme of dopamine synthesis in TIDA neurons, and detected no significant change of hypothalamic dopaminergic neurons in usp39 mutants compared with wt siblings (Figure 4D and 4H). Corticotropin releasing hormone (CRH) as well as gonadotropin-releasing hormone (GnRH) stimulates cell growth, hormone synthesis and secretion of pituitary corticotropes and gonadotropes, respectively [29,30]. However, usp39 mutants exhibit no altered crh or gnrh expression (Figure 4E–4G). Therefore, pituitary lineage expansion of usp39 mutants occurs independently of major hypothalamic neuroendocrine signals.

Pituitary transcription factors are upregulated during late stage of development in usp39 mutants

We next studied expression of transcription factors important for adenohypophyseal development. Lmx3/Lhx3 is one of the earliest pituitary specifying transcription factors and is required for progenitor proliferation and survival [2]. Pitx3, a Pitx/Rieg
Figure 3. *usp39* mutation lead to expansion of all pituitary cell lineages at 48 hpf as indicated by pituitary hormone markers. A–L: Whole-mount double *in situ* hybridization with probes indicated on the side. *usp39* mutant embryos exhibit higher expression of all pituitary hormone markers compared to wild-type (wt) embryos. Spatial distribution of *prl*, *tsh*, *pomc* and *cga* are normal in the *usp39* mutant. (A, B, E, I, M, and N) ventral view and (C, D, G, K, O, and P) lateral view, with anterior to the left. Columns 1 (A, E, I, and M) and 3 (C, G, K and O) show wt siblings; columns 2 (B, F, J, and N) and 4 (D, H, L, and P) show *usp39* mutant embryos. A–D: *gh* (purple) and *prl* (red) transcripts. (C) The spatial distribution of *gh* is normally found in the proximal pars distalis (white arrow) and *prl* is found in the rostral pars distalis (black arrow). (D) Note the spatial distribution of *gh* in the *usp39* mutant; *gh* is abnormally expressed in the rostral pars distalis (black arrow). E–H: *tsh* (purple) and *pomc* (red) transcripts. I–L: *prl* (purple) and *pomc* (red) transcripts. M–P: Whole-mount *in situ* hybridization with *cga* transcript.

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Figure 4. The hypothalamic releasing hormone and dopamine signals are unaffected in *usp39* mutants. A–C and E–G: Whole-mount *in situ* hybridization with hypothalamic probes at 48 hpf, ventral view. (A–C) wild-type (wt) embryos. (E–G) *usp39* mutant embryos. Expression of hypothalamic markers *crh*, *gnrh2*, and *gnrh3* did not change. D and H: Immunocytochemistry with tyrosine hydroxylase (TH) antibody at 48 hpf, ventral view. (H) Expression of TH in *usp39* mutant embryos did not change.

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homeodomain protein, defines the pituitary placode and is required for Lim3 expression [2]. Pit1 is a Pou domain homeoprotein and a lineage-determining factor for somatotropes, lactotropes and thyrotropes [2]. The Drosophila eye absent homolog, eya1, is required for specification of gonadotropes, corticotropes, and melanotropes [2].

Expression of these zebrafish pituitary regulators coincides within the pituitary placode of the anterior neural ridge (ANR) at 20-somite stage (18 hpf) and persists in the adenohypophysal anlage throughout 48 hpf (for eya1), or even later (for pit1, lim3, pitx3, and ascl1a). At 36 hpf, usp39 mutants exhibited no increased expression of lim3, however pit1, lim3, pitx3, eya1 and ascl1a showed a significant expression difference at a later state (48 hpf) compared with wt (Figure S1, Figure 5).

These results suggest that the usp39 mutation did not affect initial embryonic pituitary progenitor specification but induced their expansion after 36 hpf.

**usp39 mutants exhibit altered expression of cell cycle regulators including rb1 and e2f4**

To distinguish whether the altered pituitary signals detected by whole-mount in situ hybridization is due to pituitary hyperplasia or higher expression of pituitary hormone levels we crossed usp39 +/- fish to POMC-GFP transgenic fish [16]. After identifying mutant usp39 in the POMC-GFP background, we sectioned usp39 and wt whole embryos and performed immunocytochemistry with anti-GFP followed by cell number quantification. Our results demonstrated that there was an increase in the number of POMC-GFP-positive cells in usp39 mutants compared to wt (Figure S2A–S2F).

In addition, counting the nuclei stained with DAPI indicated an increase in the total number of pituitary cells in the usp39 mutant compared to wt embryos (Figure S2G). We carried out a BrdU incorporation study and demonstrated that the increase in pituitary cell number seen in usp39 mutants was due to an increase in proliferation (Figure S2H and S2I). It is well established that cell cycle dysregulation is associated with pituitary pathology in animal models and human disease [32]. However, little is known about mechanisms underlying the sensitivity of differentiated pituitary cell lineages to cell cycle regulators. We therefore performed a microarray analysis and focused on cell cycle regulators, 11 of which were confirmed for altered expression in usp39 mutants by quantitative RT-PCR. In summary, expression levels of 3 genes were increased including e2f4, rb1 (p130) and edb1a (p21) and expression of the other 8 cell cycle genes including rb1 were downregulated (Table 2; Figure S3).

We further examined e2f4 expression by RNA whole-mount in situ hybridization, which confirmed its upregulation in the adenohypophysis of usp39 mutants compared to wt embryos (Figure 6A and 6B). To investigate whether e2f4 upregulation is responsible for adenohypophysis lineage expansion, we injected embryos with antisense MO oligonucleotide to knockdown e2f4 function. The overall usp39 mutant phenotype maintained after e2f4 MO injections, which resulted in partial rescue of pOMC expression in e2f4-MO-injected usp39 embryos at 48 hpf compared to control embryos (Figure 6C–6E, mutant N = 20, ~60% showed rescue). The phenotypic rescue of usp39 embryos by e2f4-MO is pituitary specific since pOMC hypothalamic expression was not altered. In addition, we analyzed pit1 expression and a partial rescue was also observed in e2f4-MO-injected usp39 embryos (Figure 6F). These results indicate that loss of usp39 results in increased e2f4 expression, which at least partially contributes to the observed pOMC lineage expansion in zebrafish adenohypophysis.

**rb1 splicing defect contributes to the usp39 mutant phenotype**

Since usp39 is known to be an essential component of the RNA splicing machinery, the more than 70% decrease of rb1 expression in usp39 mutants may be attributed to defects in RNA splicing. We therefore examined rb1 splicing status by PCR amplification using primers corresponding to each end of 19 out of 27 exons of the rb1 gene. Primers designed for exon 3 and exon 4 resulted in a PCR product of 250 base pairs (bp) in wt and mutant embryos, representing a correctly spliced mRNA fragment. However, mutant embryos exhibited an additional larger PCR product of 343 bp (Figure 6F). Further DNA sequence analysis revealed that the 343 bp PCR product derived from usp39 mutants contains the
sequence of an unspliced intron between exon 3 and 4. The splicing defect would lead to a premature stop codon in the intron between exon 3 and exon 4 of \textit{rb1} (data not shown), which would lead to nonsense-mediated mRNA decay. We then performed an \textit{rb1} mRNA overexpression experiment in \textit{usp39} mutants and observed partial rescue of the adenohypophysis phenotype (Figure 6I, mutant N = 34, 50% showed rescue), validating the importance of \textit{usp39}-mediated \textit{rb1} mRNA splicing in controlling pituitary lineage expansion during development. In addition, we performed quantitative RT-PCR analysis on the \textit{rb1} mRNA-injected \textit{usp39} embryos and observed a 30% reduction of \textit{e2f4} expression compared to control uninjected \textit{usp39} mutants (Figure S5A), indicating that \textit{e2f4} upregulation in \textit{usp39} mutant is secondary to \textit{rb1} loss of function. Furthermore, this was confirmed by quantitative RT-PCR analysis on the \textit{e2f4} MO-injected \textit{usp39} embryos and observed that there was no change in \textit{rb1} expression compared to control uninjected \textit{usp39} mutants (Figure S5B).

### Discussion

In this study, we identified and functionally characterized the zebrafish \textit{usp39} gene, important for human and yeast pre-mRNA splicing [18,19]. We demonstrated that loss of \textit{usp39} results in defects in \textit{rb1} mRNA splicing and downregulation of \textit{rb1} expression in the pituitary gland (Table 2). We also showed that \textit{e2f4} upregulation in \textit{usp39} mutants is secondary to \textit{rb1} loss of function. These findings highlight the importance of \textit{usp39} in maintaining normal pituitary development and function.

### Table 2. Summary of cell cycle genes affected in \textit{usp39} mutant embryos from microarray hybridization and confirmed with quantitative RT-PCR.

| GB accession | Unigene ID | Location | Gene Symbol | Microarray Ratio | RT-PCR Ratio |
|--------------|------------|----------|-------------|-----------------|--------------|
| NM_213406    | Dr.75152   | chr11    | cdk2        | 0.4             | 0.18         |
| XM_689974    | Dr.13764   | chr14    | pttg        | ND              | 1.3          |
| NM_212564    | Dr.24379   | chr17    | cdc2        | 0.53            | 0.36         |
| NM_199430    | Dr.80580   | chr7     | ccnb2       | 0.58            | 0.5          |
| AF3389516    | Dr.75267   | chr25    | cdkn1b      | 0.57            | 0.53         |
| AL912410     | Dr.151578  | chr22    | cdkn1a      | ND              | 18           |
| NM_152949    | Dr.121874  | chr14    | ccna2       | 0.5             | 0.45         |
| NM_131025    | Dr.75056   | chr7     | ccnd1       | 0.82            | 0.65         |
| NM_130995    | Dr.29      | chr7     | ccne        | 0.52            | 0.54         |
| NM_213432    | Dr.77272   | chr25    | e2f4        | 2.8             | 3.09         |
| NM_001077780 | Dr.80580   | chr7     | ccnb2       | 0.58            | 0.5          |
| XM_001922133 | Dr.79466   | chr25    | rb1         | 0.49            | 0.34         |

ND indicates not determined.

**Figure 6.** Loss of \textit{usp39} leads to aberrant Rb1 mRNA splicing and increased pituitary \textit{e2f4} expression. A–B: Whole-mount double \textit{in situ} hybridization of \textit{pomc} in red and \textit{e2f4} in purple at 48 hpf, lateral view. (A) Wild-type (wt) embryo. (B) Expression of \textit{e2f4} is higher and colocalizes with \textit{pomc} expression in \textit{usp39} mutant embryos. C–E and G–I: Whole-mount \textit{in situ} hybridization of \textit{pomc} at 48 hpf, ventral view, anterior to left. (C) wt. (D) \textit{usp39} mutant. (E) \textit{e2f4} MO-injected \textit{usp39} mutant embryos showed partial \textit{pomc} rescue similar to observed in wt embryonic \textit{pomc} expression (C). F: PCR product with primers designed for region between exon 3 and exon 4 of \textit{rb1} in wt and \textit{usp39} mutant embryos. wt embryos only contain a 250 base pair (bp) PCR band, indicating that the intron was correctly spliced out. However, in the \textit{usp39} mutants there is an additional 343 bp band that contains the intron sequence. G–I: (G) wt. (H) \textit{usp39} mutant. (I) \textit{rb1} mRNA-injected \textit{usp39} mutants exhibit partial rescue of \textit{pomc} expression similar to wt embryos (G).

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expression (Figure 6F, Table 2; Figure S3). Both loss of *usp39* as well as *rb1* downregulation in *usp39* mutants may be explained by nonsense-mediated mRNA decay due to a premature termination codon. *RB1* gene mutation leading to pre-mRNA splicing defects have been shown in human cancers [35] and our study suggests a novel mechanism resulting in *rb1* splicing defects due to a *usp39* mutation.

Control of pituitary progenitor cell proliferation in concert with terminal differentiation during embryonic pituitary development is poorly understood. In mouse pituitary primordia, attenuated proliferation of cells destined to become hormone-expressing cell types occurs days before lineage-specific hormones start to express [34]. In contrast, zebrafish pituitary terminal differentiation is initiated while progenitor cells are still organized in a placodal fashion in the anterior neural ridge [2,16]. The *usp39* mutants demonstrated no early difference of adenohypophyseal primordia compared with wt, until 48 hpf when terminally differentiated cells had already migrated to a mature pituitary destination. Pituitary lineage expansion became apparent at 48 hpf in *usp39* mutants, as indicated by expression of pituitary transcription factors and lineage-specific hormone markers (Figure 3 and Figure 5). Similarly, it was found that inactivation of Rb in the small intestines of mice results in increased proliferation of differentiated cells in the villus but not in the stem cells located in the base of the crypts [35]. Therefore, our results suggest that loss of *usp39* does not affect pituitary specification, initiation and early differentiation, but does induce lineage expansion at later development stages when the cells are terminally differentiated.

Our results indicate that e2f4 overexpression has at least a partial but direct affect on adenohypophysal cell lineages in *usp39* mutants, as e2f4 antisense MO knockdown partially reverted the pituitary phenotype of *usp39* mutants (Figure 6 and Figure S4). The *usp39* mutants demonstrated persistently upregulated e2f4 expression, although molecular mechanisms leading to e2f4 overexpression remain to be determined. Overexpression of e2f4 may exert oncogenic activity promoting cell-cycle progression as previously indicated in pituitary, thyroid, lung neuroendocrine hyperplasia [36], intestinal crypt cells, colorectal cancer cells [37] as well as in prostate cancer [38]. We demonstrated an increase of POMC-GFP-positive cells in the *usp39* mutant embryos compared to wt (Figure S2). Consequently, e2f4 upregulation in *usp39* mutants may contribute to increased proliferation of terminally differentiated pituitary cells leading to lineage expansion as seen in our BrdU studies (Figure S2). On the other hand, E2F4 is a key regulator associated with p130 to promote quiescent G0 and terminal differentiation [13]. The cyclin kinase inhibitor, p21, inhibits decay of the E2F4-p130 complex, promotes senescence and restrains growth, contributing to the benign propensity of pituitary adenomas [39,40]. Our microarray and quantitative RT-PCR data showed increased *cdklna* (p21), e2f4 and *rb1* (p130), which may indicate an enhanced quiescent G0 phase inducing terminal differentiation and lineage expansion in *usp39* mutants.

Although the focus of this study was the role of *usp39* in pituitary development, this gene is also expressed in neuronal tissues and when mutated, embryos show microcephaly and smaller eyes, therefore *usp39* function may not be restricted to pituitary development. We propose that *usp39*, through targeting a set of key regulatory genes by modulating RNA splicing, should have a broader role in regulating neuronal cell lineage development. Although how *usp39* controls target mRNA splicing remains to be fully elucidated, the *usp39* ortholog of the yeast protein Ssd1p was found to have two roles: it is involved in the assembly of U4 snRNP to U6 snRNP and is also required for splicing [18]. Furthermore, previous reports have shown that a zebrafish RNA splicing factor, *p110*, is required for U4 and U6 snRNPs recycling, and a mutation in *p110* leads to thymic hypoplasia as well as eye and exocrine pancreas defects [41]. In addition, microarray analysis of *p110* mutant shows a compensatory mechanism inducing increased expression of other splicing factors, which may reverse the recycling defects [41]. We observed a similar result in our microarray analysis with upregulation of other U4/U6.U5 tri-snRNP proteins, which suggests a compensatory mechanism in *usp39* mutants (Table S2 and Figure 6F). The human tri-snRNP specific proteins include 65K, 110K and 27K are encoded by USP39, SART1 and SNRNP27, respectively and play a similar role in splicing [21]. Specifically, both *sart1* and *snrnp27* were found to be upregulated in our microarray analysis demonstrating a compensatory role due to the absence of *usp39* (Table S2). Additionally, we discovered another neuronal gene, *otx2*, which was also significantly downregulated due to a splicing defect (Figure S6). However, *otx2* mRNA overexpression in *usp39* mutant embryos did not rescue the pituitary phenotype (data not shown), validating that the Rb1/E2F4 pathway is more specific to pituitary regulation. A systematic analysis of splicing variation of all mRNA transcripts affected by *usp39* deficiency will uncover additional pathways that control neuronal and organ development by RNA splicing mechanisms.

In summary, our findings indicate that *usp39* plays an important role in pituitary development by regulating *rb1* and e2f4. Loss of *usp39* leads to pituitary cell lineage expansion through *rb1* downregulation due to a splicing defect. In addition, e2f4 overexpression contributes to increased pituitary cell mass, likely as a result of increased terminal differentiation or proliferation.

**Materials and Methods**

**Mutagenesis and fish husbandry**

Mutagenesis with ENU was performed as described [25]. Mutants including *hp689* were identified from random sibling crossing from F2 families giving rise to 25% altered *pomc* expression at 48 hpf.

**Genetic mapping**

Linkage analysis was established by mating *hp689* heterozygote in an AB background to the WIK strain. Random sibling crossing identified F1 carriers, and mutants were identified phenotypically in F2 offspring at 48 hpf. We analyzed linkage between *hp689* and simple sequence-length polymorphism markers [42]. Linkage analysis found the z34450 marker located 2.4 cM (4 recombinations in 168 meioses), G40879 marker located 0.3 cM (1 recombination in 310 meioses), G41874 marker located 0.3 cM (1 recombination in 310 meioses) in linkage group 5 (LG5) linked to the mutation.

**Genotyping of *usp39* mutants**

Total RNA derived from 48 hpf mutant and wild-type embryos was prepared by TRIZOL (Invitrogen) reagent extraction and used to generate cDNA by SuperscriptII reverse transcriptase (Invitrogen) with oligoDT primers (Roche Applied Science). The region near marker *ndrg3* contain 7 genes and sequencing the *usp39* full-length cDNA with primers CCCTTTCACAGT-GCGTTC and TTTCTTACATTGTTTACTCAGTGC from mutant embryos revealed a point mutation that converted
a TAT codon into a TAA in Exon 11, resulting in a premature termination codon rather than a tyrosine residue.

Cloning of the zebrafish usp39 cDNA

The usp39 full-length cDNA fragment was generated from wild-type embryos as described above and subcloned into pCRII-TOPO.

Morpholino, mRNA synthesis, and microinjection

Antisense MO's were injected into embryos as described [27]. The sequence of usp39 MO is 5'-TTCCGCGCTTCTGTACATATTTTAAG-3' and for e2f4 MO is 5'-ACTCTCCCATCGCTCCAGGTGTT-3' (Gene Tools, Inc.). One- to two-cell stage embryo was injected at 3.9 ng for the usp39 MO and 1.4 ng of e2f4 MO. The usp39 overexpression construct was generated by subcloning full-length usp39 cDNA from vector pCRII-usp39 into the EcoRI site of vector pXT7. The pXT7-USP39 vector was linearized with XbaI and mRNA was transcribed using T7 mMessage mMachine kit (Ambion). The T7 overexpression construct was generated by subcloning full-length rb1 cDNA (Accession Number: BC125966, OpenBiosystems) to vector pCS2+ in the StuI and XhoI sites. The rb1-pCS2+ vector was then linearized with XbaI and the mRNA transcribed using Sp6 mMessage mMachine kit (Ambion). mRNA injections were performed at the one-cell stage at approximately 200 pg for usp39 and 267 pg for rb1.

RNA in situ hybridization

Single and double whole-mount in situ hybridizations were performed as described [43]. usp39 antisense probe was synthesized from pCRII-usp39 with Sp6 RNA polymerase after linearization with NotI. The following riboprobes were generated from cDNAs as described: pome [16], gh, pit1, tdh, lim3, pit1 and pitx3 [2], eya1 [44], eph [29], gnrh2 and gnrh3 [30], and ascl1a [31]. Full-length cDNA for ega (Accession Number: BC116611) and e2f4 (Accession Number: BC056832) were purchased from OpenBiosystems. e2f4 was subcloned to pCRII-TOPO vector and linearized with SpeI whereas the ega-Express1 was linearized with EcoRI and riboprobes were synthesized with T7 RNA polymerase.

Antibody staining

Whole-mount antibody staining was performed using a rabbit anti-tyrosine hydroxylase (TH) primary antibody at 1:200 dilution (Chemicon) and detected with an Alexa (A594)-conjugated goat anti-tyrosine hydroxylase (TH) primary antibody at 1:200 dilution (Invitrogen). A drop of ProLong Gold antifade reagent with DAPI (Invitrogen) was added to sections and coverslipped. (A, B) All pituitary POMC-GFP-positive cells are in the same sections of the four wt and four usp39 mutants. (C–E) Three sections are required to include all pituitary POMC-GFP positive cells in usp39 mutant embryos. (F) Four wt and four usp39 mutants were analyzed for POMC-GFP-positive cells. usp39 mutants had an average of 25.35 POMC-GFP-positive cells compare to 12.5 in wt embryos (mean ± SEM; p<0.02, n = 4). (G) To determine the total number of pituitary cells, DAPI positive cells were counted in the same sections of the four wt and four usp39 mutants. usp39 mutants had an average of 123.5 DAPI-positive cells in the pituitary compare to 84.25 in wt embryos (mean ± SEM; p<0.03, n = 4). (H–I) Embryos were placed in 10 mM solution of BrdU (Invitrogen) in fish water at 10-somite stage and kept in dark until 48 hpf. The embryos were also injected with 10 nl of 10 mM BrdU at 24 hfp. BrdU labeling results in 48 hpf embryos by immunocytochemistry using anti-BrdU antibody and anti-BrdU was performed in whole-mount embryos as described in [Liu et al.]. The anti-BrdU, rabbit IgG fraction and Alexa Fluor 488 secondary antibody are at 1:200 dilution (Invitrogen). The anti-BrdU, mouse IgG fraction (Santa Cruz) and Alexa Fluor 594 secondary antibody (Invitrogen) are at 1:100 and 1:200 dilution, respectively. Imaging was performed on variobte 100 μm sections of wt and usp39 mutant embryos. (H) Proliferating cells in the pituitary were not evident in wt embryos. (I) usp39 mutant embryos contain an expression change in usp39 mutants over WT embryos ± standard error (for primer sequences see Table S1).

Retinoblastoma splicing primers

cDNA was generated as described above. We designed primers that covered the exon and intron region of Exon 3 to Exon 4 of the rb1 gene. The primers used were: CGGTATTGCAACAGA-CAGCA and GGTAGAGGGCGCAATGTCACA.

Vibratome sections

After whole-mount in situ hybridization, embryos were washed in PBS, manually deyolled, and mounted on their lateral side in 4% low melting agarose (Fisher Scientific) in PBS. Thin 100 μm slices were cut using a vibratome (Vibratome 1000 Plus) and sections were stored in PBS until imaging.

Image acquisition and processing

The in situ hybridization and the vibratome sections were imaged with an AxioCam digital camera (Zeiss) mounted on an Axioplan 2 compound microscope (Zeiss). OpenLab 4.0.2 software (Improvement) was used to capture all images; Photoshop CS4 software (Adobe Systems) was used for further image processing.

Supporting Information

Figure S1  lim3 expression is not altered at 36 hpf. (A, B) Whole-mount in situ hybridization with lim3 probe, ventral view with anterior to left. (A) wild-type (wt); (B) Expression of lim3 in usp39 mutant embryos is not changed compared to wt embryos at 36 hpf. Found at: doi:10.1371/journal.pgen.1001271.s001 (1.30 MB TIF)

Figure S2  Increase in pituitary cell number in usp39 mutants compared to wt embryos. Immunocytochemistry using anti-GFP antibody was performed on 5 μm frontal sections of wt and usp39 mutants with a POMC-GFP transgenic background at 48 hpf, ventral view, anterior on top. The anti-GFP, rabbit IgG fraction, Alexa Fluor 488 conjugated antibody are at 1:200 dilution (Invitrogen). A drop of ProLong Gold antifade reagent with DAPI (Invitrogen) was added to sections and coverslipped. (A, B) All pituitary POMC-GFP-positive cells are in the same sections of the wild-type embryos. (C–E) Three sections are required to include all pituitary POMC-GFP positive cells in usp39 mutant embryos. (F) Four wt and four usp39 mutants were analyzed for POMC-GFP-positive cells. usp39 mutants had an average of 25.35 POMC-GFP-positive cells compare to 12.5 in wt embryos (mean ± SEM; p<0.02, n = 4). (G) To determine the total number of pituitary cells, DAPI positive cells were counted in the same sections of the four wt and four usp39 mutants. usp39 mutants had an average of 123.5 DAPI-positive cells in the pituitary compare to 84.25 in wt embryos (mean ± SEM; p<0.03, n = 4). (H–I) Embryos were placed in 10 mM solution of BrdU (Sigma) in fish water at 10-somite stage and kept in dark until 48 hpf. The embryos were also injected with 10 nl of 10 mM BrdU at 24 hfp. BrdU labeling results in 48 hpf embryos by immunocytochemistry using anti-BrdU antibody and anti-BrdU was performed in whole-mount embryos as described in [Liu et al.]. The anti-BrdU, rabbit IgG fraction and Alexa Fluor 488 secondary antibody are at 1:200 dilution (Invitrogen). The anti-BrdU, mouse IgG fraction (Santa Cruz) and Alexa Fluor 594 secondary antibody (Invitrogen) are at 1:100 and 1:200 dilution, respectively. Imaging was performed on vibratome 100 μm sections of wt and usp39 mutant embryos. (H) Proliferating cells in the pituitary were not evident in wt embryos. (I) usp39 mutant embryos contain an expression change in usp39 mutants over WT embryos ± standard error (for primer sequences see Table S1).
higher number of proliferating cells in the pituitary region marked by white arrowheads. [Liu NA, Ren M, Song J, Rios Y, Wawrowsky K, et al. (2008) In vivo time-lapse imaging delineates the zebrafish pituitary protopiamelanocortin lineage boundary regulated by FGF3 signal. Dev Biol.]

Found at: doi:10.1371/journal.pgen.1001271.s005 (9.49 MB TIF)

**Figure S3** Quantitative RT-PCR analysis showing expression of cell cycle regulators with loss of usp39. Relative gene expression levels were calculated for usp39 mutant embryos compared to control WT embryos (see Materials and Methods). Dashed line represents a value of 1, which corresponds to no change in WT expression. Significant change was determined by two standard deviations (Mean ± SEM).

Found at: doi:10.1371/journal.pgen.1001271.s003 (9.64 MB TIF)

**Figure S4** e2f4 morpholino injections rescued *prl* expression in usp39 mutants. (A–C) Whole-mount in situ hybridization with *prl* probe, ventral view with anterior to left. (A) wt. (B) *usp39* mutant embryo. (C) Expression of *prl* in e2f4-MO-injected *usp39* embryos was partially rescued.

Found at: doi:10.1371/journal.pgen.1001271.s004 (9.55 MB TIF)

**Figure S5** e2f4 mRNA expression is partially rescued by *rb1* mRNA overexpression in *usp39* mutant embryos while e2f4 MO injections did not rescue *rb1* mRNA levels. A 30% decrease of e2f4 expression was detected in the *rb1* mRNA injected *usp39* embryos compared to control uninjected *usp39* mutants by quantitative RT-PCR (mean ± SEM; n = 60 embryos; p<0.03). (B) However, the expression of *rb1* mRNA levels by quantitative RT-PCR of *usp39* mutants injected with e2f4 morpholino did not change (mean ± SEM; n = 67 embryos).

Found at: doi:10.1371/journal.pgen.1001271.s008 (0.04 MB TIF)

**Figure S6** *otx2* splicing defect in *usp39* mutants. (A, B) Whole-mount in situ hybridization of *otx2* at 48 hpf, ventral view, anterior to left. (A) wt. (B) Expression of *otx2* is downregulated in *usp39* mutants. (C) PCR product with primers designed for a region between exon 3 and exon 4 of *otx2* in wt and *usp39* mutant embryos. In addition to the 79 bp band in wt, the *usp39* mutant embryos contain an additional 289 bp band, which corresponds to a misspliced mRNA fragment including the intron between exon 3 and 4. The primers used were: GGCCCTGAAATCACCCTGC and CTGCTGTGGGGACACTTTT.

Found at: doi:10.1371/journal.pgen.1001271.s007 (0.05 MB TIF)

**Table S1** Quantitative RT-PCR primers.

Found at: doi:10.1371/journal.pgen.1001271.s001 (0.05 MB DOC)

**Table S2** Tri-sRNP factors affected in *usp39* mutants.

Found at: doi:10.1371/journal.pgen.1001271.s008 (0.04 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: YR NAL SL. Performed the experiments: YR NAL SL. Analyzed the data: YR SM NAL SL. Wrote the paper: YR NAL SL.

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