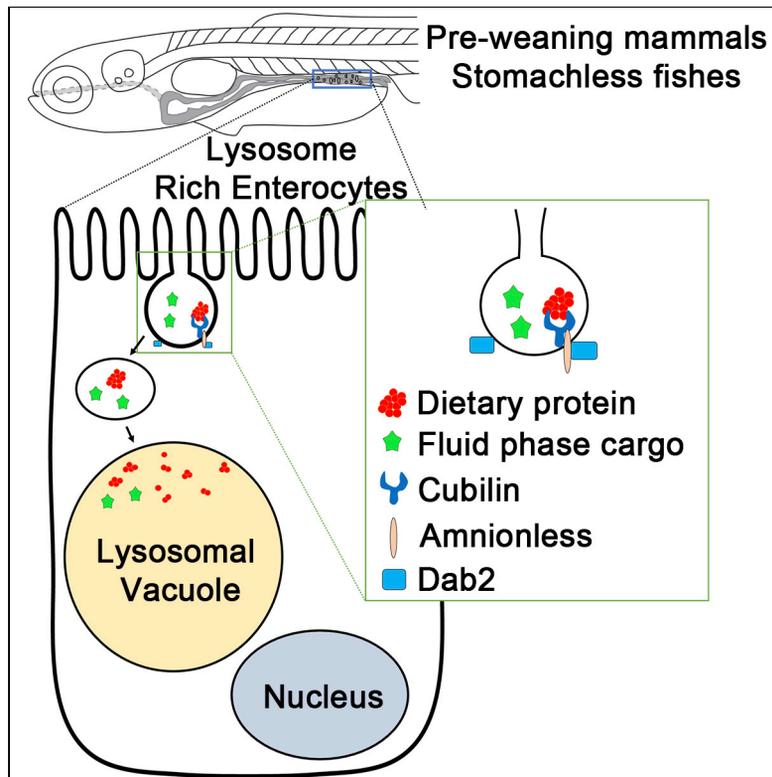


# Developmental Cell

## Lysosome-Rich Enterocytes Mediate Protein Absorption in the Vertebrate Gut

### Graphical Abstract



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### In Brief

Lysosome-rich enterocytes (LREs) are highly endocytic enterocytes found in pre-weaning mammals and stomachless fishes. Park et al. report that LREs mediate dietary protein absorption in the immature vertebrate gut via a multi-ligand endocytic machinery, composed of Cubilin, Amnionless, and Dab2, and a large lysosomal vacuole where dietary proteins are digested.

### Highlights

- Lysosome-rich enterocytes (LREs) internalize and digest dietary protein intracellularly
- LREs are conserved between zebrafish and mammals
- Cubn, Amn, and Dab2 mediate high-capacity protein uptake in LREs
- Loss of LRE function impairs growth and survival in zebrafish and mice

# Lysosome-Rich Enterocytes Mediate Protein Absorption in the Vertebrate Gut

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## SUMMARY

The guts of neonatal mammals and stomachless fish have a limited capacity for luminal protein digestion, which allows oral acquisition of antibodies and antigens. However, how dietary protein is absorbed during critical developmental stages when the gut is still immature is unknown. Here, we show that specialized intestinal cells, which we call lysosome-rich enterocytes (LREs), internalize dietary protein via receptor-mediated and fluid-phase endocytosis for intracellular digestion and trans-cellular transport. In LREs, we identify a conserved endocytic machinery, composed of the scavenger receptor complex Cubilin/Amnionless and Dab2, that is required for protein uptake by LREs and for growth and survival of larval zebrafish. Moreover, impairing LRE function in suckling mice, via conditional deletion of Dab2, leads to stunted growth and severe protein malnutrition reminiscent of kwashiorkor, a devastating human malnutrition syndrome. These findings identify digestive functions and conserved molecular mechanisms in LREs that are crucial for vertebrate growth and survival.

## INTRODUCTION

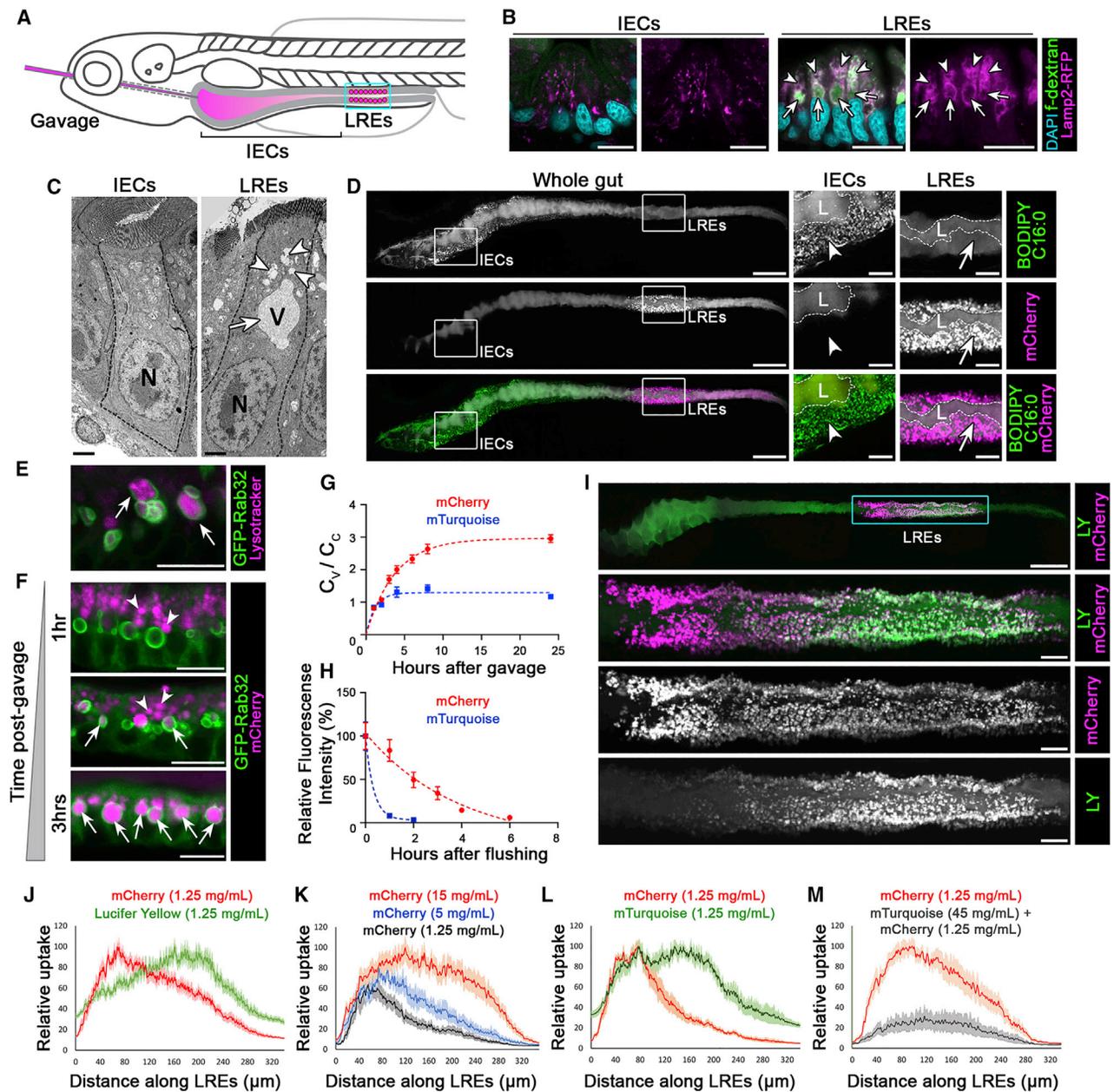
Protein is an essential macronutrient for organismal growth and health. Protein digestion in weaned mammals occurs in the lumen of gastrointestinal (GI) tract by the action of pepsin in the stomach, followed by various proteases in the intestinal lumen. This produces oligopeptides and amino acids that are then taken up by enterocytes via specific transporters (Henning, 1985). By contrast, in the GI tract of zebrafish and suckling stage mammals, proteins are not fully digested in the lumen because of low protease activity (Henning, 1985; Robberecht et al., 1971; Rombout et al., 1985; Zhang et al., 2005). This allows antigens and maternal

antibodies to be preserved and thus contribute to the development of innate and adaptive immune systems (Kulkarni and Newberry, 2019; Reinhardt, 1984). However, this feature of the immature vertebrate gut also presents a nutritional challenge and highlights the need for an alternative mechanism mediating dietary protein absorption. Despite its critical importance in organismal development and survival, how dietary protein absorption occurs in the immature vertebrate gut remains unclear.

One possibility is that when the GI tract is still immature, dietary protein is internalized and digested intracellularly by intestinal epithelial cells. All eukaryotic cells internalize membrane, protein cargo, and fluid from the extracellular medium. Some cargoes such as growth factors are recognized by specific receptors that enable their uptake (i.e., receptor-mediated endocytosis), while other proteins and soluble cargoes are simply internalized along with the fluid in a non-specific fashion (i.e., fluid-phase endocytosis). Fluid-phase endocytosis, also known as pinocytosis, can occur as a byproduct of receptor-mediated endocytosis or via endocytic pathways such as caveolae internalization and various forms of clathrin-independent endocytosis that are not fully understood (Johannes et al., 2015).

Classic tracing and electron microscopy (EM) experiments suggested that proteins present in the lumen of stomachless fish and immature mammalian GI tract can be internalized by a subpopulation of intestinal cells known as vacuolated enterocytes (Kleinman and Walker, 1984) via non-specific fluid-phase endocytosis and membrane adsorption (Gonnella and Neutra, 1984; Graney, 1968; Rombout et al., 1985). However, it is unclear if this activity is specific for proteins or sufficient in volume to be of nutritional importance.

Vacuolated enterocytes can be found in the ileum of pre-weaning mammals and the homologous intestinal region of zebrafish and other fishes (Harper et al., 2011; Kraehenbuhl and Campiche, 1969; Lickwar et al., 2017; Ng et al., 2005; Rodríguez-Fraticelli et al., 2015; Wallace et al., 2005). In mammals, vacuolated enterocytes are replaced by mature enterocytes at weaning, and premature replacement results in growth retardation and elevated neonatal mortality (Harper et al., 2011; Muncan et al., 2011). In contrast, zebrafish vacuolated enterocytes persist into adulthood (Lickwar et al., 2017; Ng et al., 2005;



**Figure 1. Luminal Proteins Are Internalized and Digested Intracellularly by LREs in the Zebrafish Intestine**

(A) Cartoon depicting gavage approach for studying luminal cargo uptake into LREs (cyan box) in the larval zebrafish intestine.

(B) Confocal images of cross sections of intestinal segments showing IECs and LREs from a 6 dpf larva following gavage with fluorescent dextran (fDex). Lamp2 is highly expressed in LREs and localizes to endosomes (arrowheads) and lysosomal vacuoles (arrows) that accumulate internalized fDex.  $n \geq 10$  animals. Scale bars, 10  $\mu\text{m}$ .

(C) EM images of an IEC and an LRE. Arrowheads mark endosomes and an arrow the lysosomal vacuole. N = nucleus; V = vacuole.  $n \geq 5$  animals. Scale bars, 2  $\mu\text{m}$ .

(D) Live confocal images of a 6 dpf larval zebrafish intestine following gavage and internalization of BODIPY C16:0 and mCherry. IECs take lipids and LREs protein. L = lumen. Insets show magnified images marking lipid droplets (arrowheads) in IECs and lysosomal vacuoles in LREs (arrows).  $n = 5$ . Scale bars, 100  $\mu\text{m}$  (whole gut images), 25  $\mu\text{m}$  (magnified images).

(E) Live confocal images of LREs of 6 dpf larva expressing GFP-Rab32a 1 h after gavage with LysoTracker, which labels vacuoles (arrows).  $n \geq 10$  animals. Scale bar, 10  $\mu\text{m}$ .

(F) Live confocal images of LREs of 6 dpf larvae expressing GFP-Rab32a that were gavaged with mCherry. Luminal protein (mCherry) was internalized, migrated through apical endosomes (arrowheads), and progressively accumulated in LRE vacuoles (arrows). Scale bars, 10  $\mu\text{m}$ .

(G) Quantitation of mCherry and mTurquoise accumulation in LRE vacuoles ( $C_v$ ) respect to total cellular mean pixel intensity ( $C_c$ ) over time after gavaging with 1.25 mg/mL of mCherry or mTurquoise. Data are fitted with the model  $C_v/C_c = k_{in}/k_{ex} * (1 - \exp(-k_{ex} * X))$  where  $k_{in}$  is the vacuole internalization rate and  $k_{ex}$  is the degradation rate.  $n \geq 28$ ; LREs from at least 6 animals per timepoint.

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Wang et al., 2010). Notably, previous work showed that reduction in the number and endocytic activity of vacuolated enterocytes, due to loss of Pllp function, impairs larval survival under nutrient limiting conditions (Rodríguez-Fraticelli et al., 2015). Together, these studies suggested that vacuolated enterocytes have an important function in zebrafish and pre-weaning mammals. However, whether vacuolated cells play a nutritional role, what types of nutrients they absorb, and what cellular mechanisms control their physiological activity are unknown.

Here, we used a comprehensive approach to elucidate the cellular and molecular mechanism mediating protein absorption in the immature gut of zebrafish and suckling mice. We show that vacuolated enterocytes, which we define as lysosome-rich enterocytes (LREs), preferentially internalize dietary proteins both via receptor-mediated and fluid-phase endocytosis and digest them intracellularly. We identify a multi-ligand endocytic machinery, composed of Cubilin (Cubn), Amnionless (Amn) and Dab2, that mediates the endocytic activity of LREs. Using custom diets and CRISPR/Cas9-generated mutants, we show that LRE-mediated protein absorption is crucial for larval zebrafish growth and survival. Conditional deletion of Dab2 in pre-weaning mice impairs protein absorption in LREs, leading to severe malnutrition and stunted growth, showing that LREs are morphologically and functionally conserved between zebrafish and suckling mammals.

## RESULTS

### Luminal Proteins Are Internalized and Digested Intracellularly by LREs in the Zebrafish Intestine

To investigate the function of vacuolated enterocytes in the larval zebrafish gut, we gavaged animals with fluorescent dextran (fDex) (Cocchiario and Rawls, 2013). After gavage, we observed a population of cells in the posterior mid-intestine that avidly internalize fDex and found that these cells express high levels of the lysosomal marker Lamp2 and possess a large supranuclear vacuolar system (Figures 1A–1C). This is in contrast to other intestinal epithelial cells (IECs) that have smaller lysosomes scattered throughout the cell (Figure 1B). We therefore named these vacuolated intestinal cells lysosome rich enterocytes (LREs).

To define the luminal contents internalized by LREs, we gavaged 6 days post-fertilization (dpf) larvae with a mixture of purified mCherry (Shaner et al., 2004) protein and fluorescently labeled fatty acid (Semova et al., 2012) and imaged cargo uptake throughout the entire gut after 1 h using live confocal microscopy. We observed that while both fluorescent cargoes were present along the length of the lumen, LREs internalized mCherry but not fatty acids, which were instead absorbed by IECs in the anterior gut as previously shown (Carten et al., 2011) (Figure 1D). We then confirmed that the vacuolar compartments in LREs

were bona fide lysosomes by labeling experiments with the acidotropic dye LysoTracker (Figure 1E). Moreover, the vacuole membranes were lined by GFP-Rab32a, which is also found at the membrane of notochord vacuoles, a large lysosome related organelle (Ellis et al., 2013) (Figure 1E).

Following gavage, mCherry was rapidly internalized from the lumen into apical endosomes and late endosomes (Figure 1F, arrowheads and Figures S1A–S1E), accumulating progressively in vacuoles (Figure 1F, arrows). To investigate the kinetics of this process, we gavaged mCherry or mTurquoise, whose fluorescence is pH insensitive (Shinoda et al., 2018), and measured their accumulation within lysosomal vacuoles, lined by GFP-Rab32a, relative to total cellular signal (Cv/Cc) over time. mCherry and mTurquoise reached their half maximal saturation within  $2.6 \pm 0.2$  h and  $0.8 \pm 0.2$  h, respectively, and subsequently reached an equilibrium between their rate of import from the luminal pool and their degradation in the vacuole (Figure 1G). To assess the degradation rate of each protein in LREs, we gavaged mCherry or mTurquoise and allowed their uptake for 1 h. Then, we flushed the luminal protein pool by gavaging PBS and measured the fluorescence intensity of each protein in LREs over time. While mTurquoise was quickly degraded (0.23 h half-life) and was essentially gone by 2 h post-flushing, mCherry had a much longer half-life (2.7 h) and was detectable in LREs 5 h post-flushing, which is consistent with the relative resistance to proteolysis reported for mCherry (Shinoda et al., 2018) (Figure 1H). These data show that LREs continuously internalize proteins from the lumen and digest them intracellularly.

To assess internalization of labeled cargoes by LREs, we first gavaged mCherry and the water-soluble and small molecular weight cargo lucifer yellow (LY) (Swanson et al., 1985) into two transgenic fish lines that allow visualization of the apical membrane (Figures S1F–S1K). We quantified mCherry and LY uptake in LREs and found that both were efficiently internalized by these cells. However, the peaks of absorption were slightly shifted, with mCherry concentrated at the anterior of the LRE segment (Figures 1I and 1J). When we analyzed mCherry internalization using increasing concentrations of the protein, we found that its uptake profile shifted to the posterior of the LRE region, similar to LY, suggesting that mCherry absorption in anterior LREs is saturable (Figure 1K). Additional experiments using other pH insensitive protein cargoes revealed distinct internalization profiles for each protein (Figures 1L and S2A). When we gavaged mCherry together with an excess of mTurquoise, uptake of mCherry was impaired (Figure 1M) and vice versa (Figure S2D), suggesting competition for the same internalization machinery. Similar results were obtained using mCherry and labeled transferrin (Figures S2B and S2C). These data suggest that protein

(H) Quantitation of mCherry and mTurquoise degradation in LREs over time following gavage with 1.25 mg/mL mCherry or 5 mg/mL mTurquoise for 1 h. Data are fitted with one phase decay model (dotted lines).  $n \geq 9$  animals per timepoint.

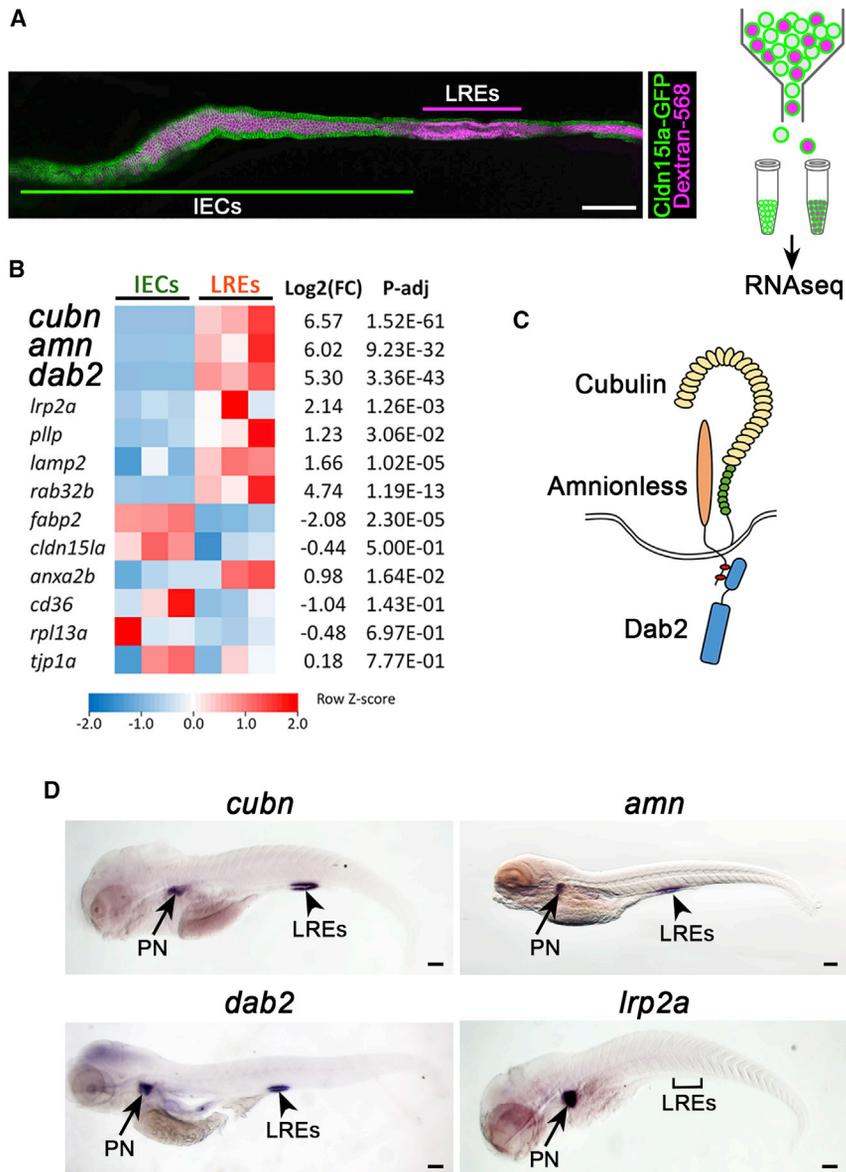
(I) Live confocal image of a 6 dpf larva gavaged with lucifer yellow (LY) and mCherry. Cyan box indicates magnified inset of LREs in lower panels. Scale bar, 100  $\mu$ m (whole gut image), 20  $\mu$ m (magnified images).

(J) Internalization profiles of LY and mCherry along LREs ( $n = 12$ ).

(K) Internalization profiles along LRE region following gavage with increasing concentrations of mCherry: 1.25 mg/mL ( $n = 7$ ), 5 mg/mL ( $n = 8$ ), 15 mg/mL ( $n = 9$ ).

(L) Internalization profiles along LRE region of mCherry and mTurquoise ( $n = 8$ ).

(M) Internalization profiles along LRE region of mCherry following gavage with either mCherry alone ( $n = 7$ ) or both mCherry + excess mTurquoise to outcompete mCherry uptake ( $n = 7$ ). Data are means  $\pm$  S.E.M. in (G), (H), and (J–M). Whole gut images in (D) and (I) are digitally stitched. See also Figures S1–S3.



**Figure 2. LREs Specifically Express a Multi-ligand Endocytic Machinery Composed of Cubn, Amn, and Dab2**

(A) Isolation and analysis of gene expression profiles in LREs versus IEC. 6 dpf larvae expressing Cldn15la-GFP throughout the gut were gavaged with alexa-568 dextran (left panel) and subjected to FACS to isolate IECs and LREs for RNA-seq (right panel). Scale bar, 100  $\mu$ m.

(B) Heatmap of RNA-seq data depicting expression levels of selected genes in LREs and IECs. Three independent samples for each cell population were used for RNA-seq experiment.

(C) Cartoon illustrating the multi-ligand endocytic machinery composed of Cubn, Amn, and Dab2.

(D) *In situ* hybridization of *cubn* (cubilin), *amn* (amnionless), *dab2*, and *lrp2a* (megalin). *cubn*, *amn*, and *dab2* transcripts are enriched in LREs and pronephros. Bracket marks LRE region devoid of *lrp2a* transcript expression. PN = pronephros. n  $\geq$  20 animals, Scale bar, 100  $\mu$ m. See also Figures S4 and S5.

(Figures 2A and S4A). Following RNA-seq analyses, we found that LREs showed increased expression of *pllp* and *lamp2*, consistent with previous results (Rodríguez-Fraticelli et al., 2015), and reduced expression of IEC markers such as *ifabp2* and *mttf* (Figures 2B and S4B). Differential expression analysis, cluster dendrogram, and principal-component analysis confirmed that each population had unique enrichment signatures (Figures S4C–S4E). Profiling using the Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the terms associated with lysosomal activity including lysosome, proteolysis, and peptidase activity were highly enriched in LREs (Figure S4F), which is consistent with the presence of a prominent lysosomal degradative system in

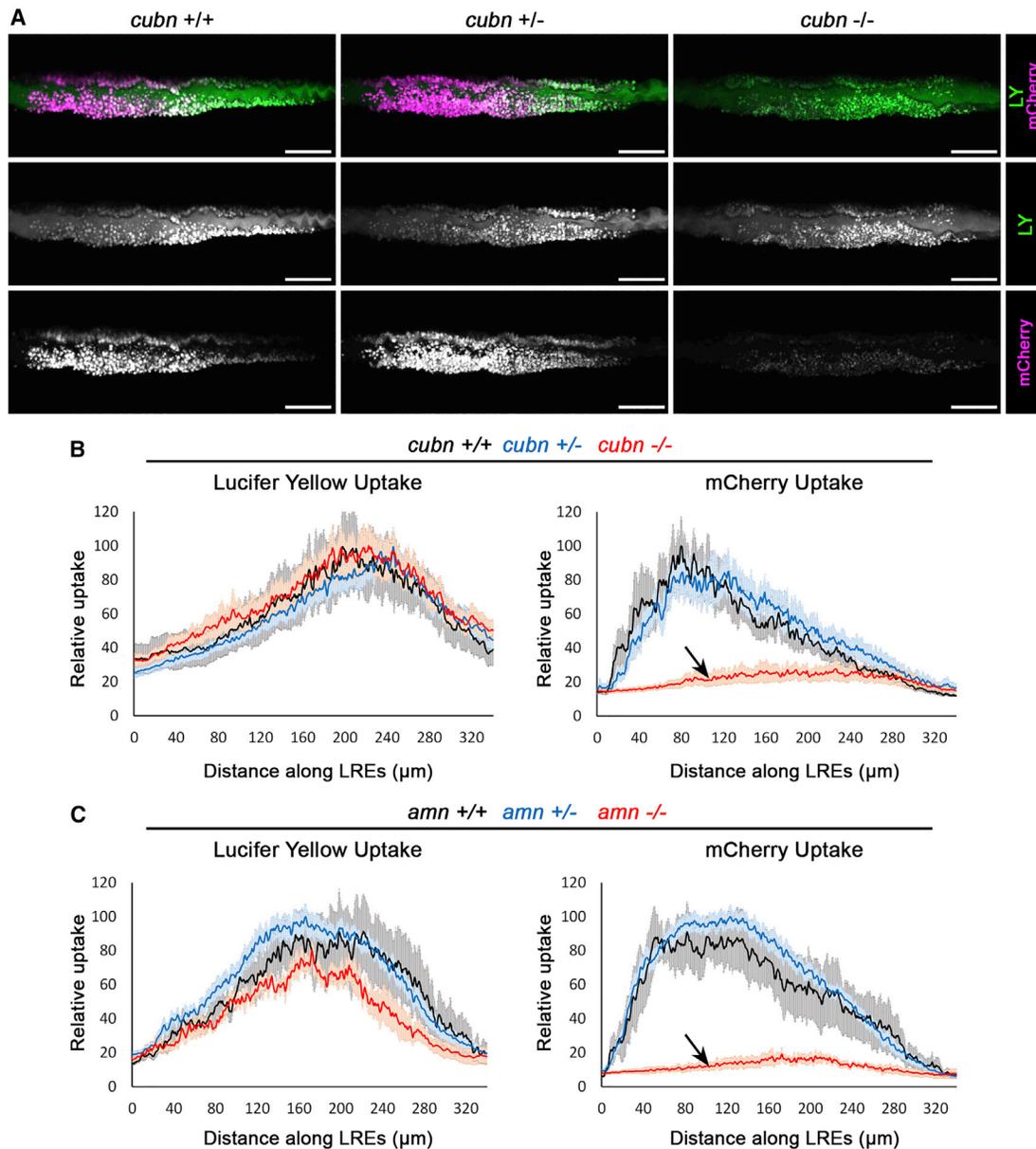
uptake in LREs is mediated by a saturable mechanism of broad specificity and variable affinity, as the internalization peaks are slightly different for each protein we tested. We also found that a fraction of internalized fluorescent proteins and LY was later found in the pronephros (kidney), liver, and gall bladder (Figures S3A and S3B), suggesting that one function of LREs is to mediate transcellular transport of proteins and soluble cargoes across the intestinal barrier and subsequently to other tissues.

**LREs Specifically Express a Multi-ligand Endocytic Machinery Composed of Cubn, Amn, and Dab2**

To identify the molecular mechanisms mediating protein uptake in LREs, we gavaged fDex into 5 dpf *TgBAC(cldn15la-GFP)* transgenic larvae in which Cldn15la-GFP localizes to the lateral surface of all intestinal cells (Alvers et al., 2014). IECs (GFP only) and LREs (GFP + fDex) were isolated using fluorescence-activated cell sorting (FACS) 24 h after gavage

these cells. Among the most highly enriched LRE transcripts were *cubn*, *amn*, and *dab2* (Figure 2B).

Cubn is a large, broad-spectrum scavenger receptor previously shown to be involved in protein retrieval in the kidney and absorption of intrinsic factor-vitamin B12 in the intestine (Amsellem et al., 2010; Nielsen et al., 2016). In the mouse embryonic visceral yolk sac endoderm, Cubn has also been implicated in the absorption of nutrients including folic acid, transferrin, retinoic acid, and cholesterol (Kozyraki and Gof-flot, 2007; Zohn and Sarkar, 2010). Amn forms a large complex with Cubn (Larsen et al., 2018), where Amn functions as a transmembrane link for Cubn and provides intracellular binding sites for the endocytic adaptor Dab2 (Fyfe et al., 2004; He et al., 2005; Maurer and Cooper, 2005; Strophe et al., 2004) (Figure 2C). *In situ* hybridization for the respective transcripts revealed that *cubn*, *amn*, and *dab2* are expressed in a highly specific manner in the LRE region of the gut and in



### Figure 3. The Cubn/Amn Endocytic Receptor Complex Mediates Protein Uptake in LREs

(A) Live confocal images showing LY and mCherry uptake in LREs of *cubn*<sup>pd1169</sup> +/+, +/-, -/- 6 dpf larvae. Larvae were imaged 5 h after gavage. *cubn*<sup>pd1169</sup> mutants show selective loss of protein (mCherry) internalization. Scale bar, 50  $\mu$ m.

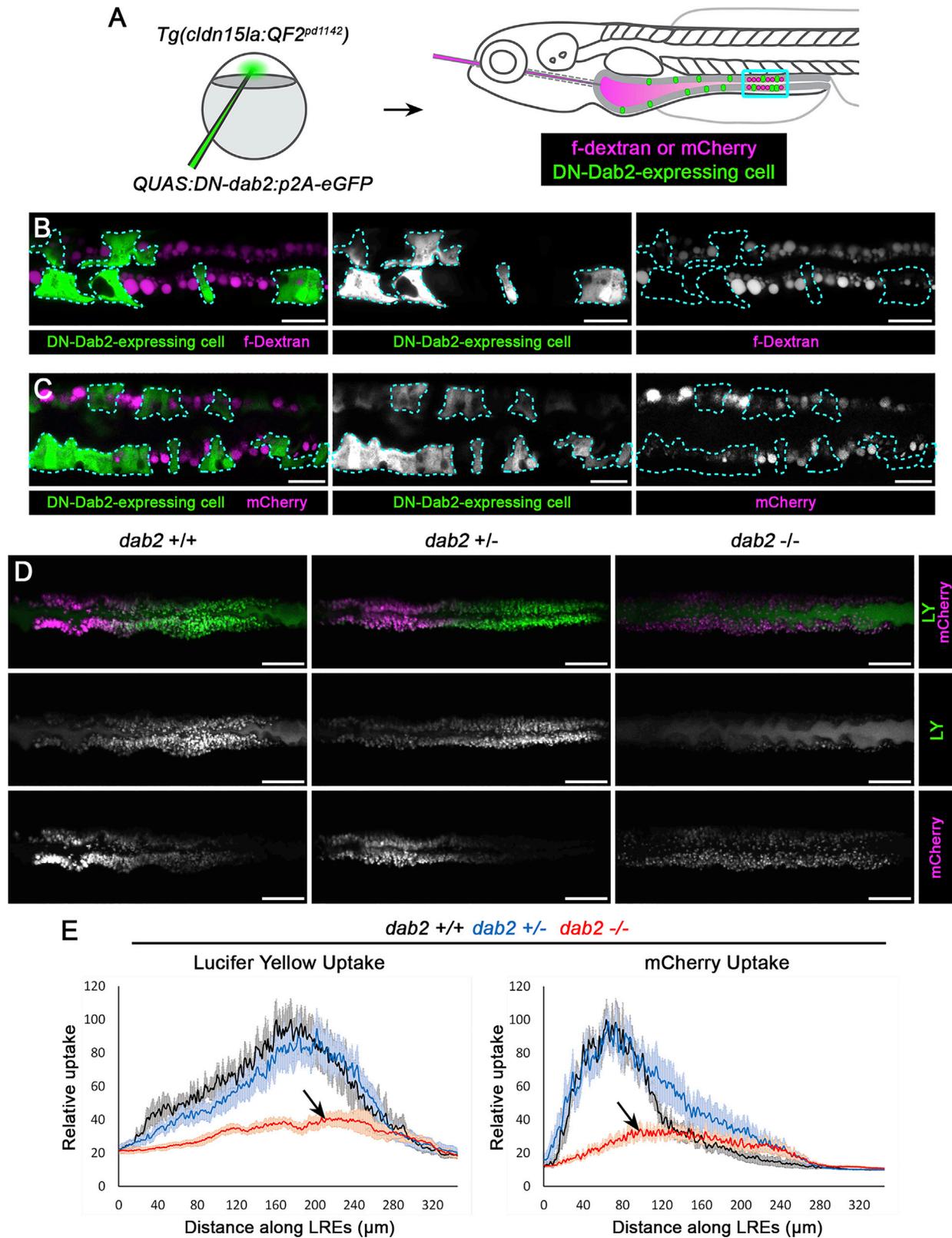
(B and C) Internalization profiles of LY (left) and mCherry (right) of 6dpf *cubn*<sup>pd1169</sup> +/+ (n = 5), +/- (n = 10), -/- (n = 9) (B) and of *amn*<sup>pd1169</sup> +/+ (n = 4), +/- (n = 15), -/- (n = 4) (C). Endocytic receptor mutants *cubn* and *amn* exhibited specific defects in protein uptake (arrows). Data are means  $\pm$  S.E.M. See also [Figures S5](#) and [S6](#).

the pronephros ([Figures 2D](#), [S5A](#), and [S5B](#)). In contrast, *Irp2a* (a.k.a. megalin), which encodes another scavenger receptor, was undetectable in LREs by *in situ* hybridization and instead showed specific expression in the pronephros ([Figure 2D](#)). Together, these data suggest that Cubn/Amn and Dab2 mediates saturable uptake of proteins in LREs.

### The Cubn/Amn Endocytic Receptor Complex Mediates Protein Uptake in LREs

To test the role of Cubn in LRE protein uptake, we generated a mutant zebrafish line using CRISPR/Cas9 ([Figures S5C–S5E](#))

and monitored mCherry/LY uptake as before. Interestingly, while mCherry uptake was significantly impaired, LY internalization was unaffected in *cubn* mutants ([Figures 3A](#) and [3B](#)). Similar results were obtained using mTurquoise and transferrin (tfn) as protein cargoes ([Figure S2E](#)), suggesting Cubn mediates the uptake of a broad spectrum of proteins, but not small soluble compounds such as LY. The residual (~20%) uptake of tfn remaining in *cubn* mutants ([Figure S2E](#), asterisk) was likely mediated by its cognate receptor, the tfn receptor, which is expressed in LREs according to our RNA-seq data. To test if Cubn function depends on Amn as shown in other contexts



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(Fyfe et al., 2004; He et al., 2005), we also generated *amn* mutants (Figures S5F–S5H) and assayed LRE activity. mCherry, but not LY, uptake was significantly impaired upon loss of *Amn* function (Figure 3C). These data indicate that protein uptake in LREs is largely mediated by the Cubn/*Amn* complex in zebrafish.

### Dab2 Mediates Fluid-Phase and Receptor-Dependent Endocytosis in LREs

To test the role of Dab2 in cargo uptake from the intestinal lumen, we first isolated the phosphotyrosine binding (PTB) domain of Dab2, which acts as a dominant-negative (DN) (Mishra et al., 2002), and expressed it mosaically in the intestine under the control of QUAS (Subedi et al., 2014), using *cldn15la:QF2* as a driver (Figure 4A). Using fDex and mCherry as cargoes, we found that uptake was impaired in cells expressing DN-Dab2 in a cell autonomous manner (Figures 4B and 4C). We then generated a *dab2* zebrafish mutant using CRISPR/Cas9 (Figures S5I–S5K) and assayed mCherry/LY uptake, confirming that uptake of both receptor-dependent and fluid-phase cargoes in LREs require Dab2 function (Figures 4D and 4E). Thus, Dab2 function is critical for the robust ability of LREs to internalize protein and other soluble cargoes. In contrast to protein and other soluble cargo uptake, fatty acid uptake in IECs was unaffected by loss of Cubn/*Amn* or Dab2 function (Figures S6A–S6D). We also examined lysosomal vacuole formation in LREs and found that it occurs normally upon loss of Cubn/*Amn* or Dab2 function (Figures S6E and S6F), indicating that formation of the large lysosomal compartment of LREs is not dependent on their internalization activity.

### Loss of LRE Protein Uptake Impairs Growth and Survival in Larval Zebrafish

Our data indicate that protein uptake in the zebrafish intestine occurs via the Cubn/*Amn* receptor complex, which is internalized via the Dab2 endocytic adaptor. Our data also indicate that fluid-phase endocytosis is dependent on the activity of the Dab2 adaptor, but not on Cubn/*Amn* receptor complex. In previous work we found that loss of Pllp, which interacts with Dab2 in MDCK cells (Rodríguez-Fraticelli et al., 2015), partially impairs LRE differentiation and reduces larval survival under nutrient limiting conditions (Rodríguez-Fraticelli et al., 2015). Thus, we investigated whether LREs play a role in dietary protein absorption in zebrafish. First, we generated nearly isocaloric custom diets with high protein (HP) or low protein (LP) content. Then, we raised *cubn* and *dab2* mutants and their heterozygous control larvae under a calorie-restricted HP or LP feeding regimen as well as a non-calorie-restricted standard control feeding regimen. Under those conditions, *cubn* mutants survived normally when fed a standard control diet or a calorie-restricted

HP diet, but showed poorer survival rate under a calorie-restricted LP diet (Figure 5A). On the other hand, *dab2* mutants presented a sharp and significant reduction in survival relative to their heterozygous controls under all feeding conditions (Figure 5B). We also found that both *cubn* and *dab2* mutants fed a standard control diet were growth-stunted (Figure 5C) because of poor growth during larval stages (Figure 5D). In addition to strong survival and growth defects, *dab2* mutants presented a poorly folded and shorter, but well polarized epithelium compared to heterozygous controls (Figure 5E), which is typically observed in starved larvae.

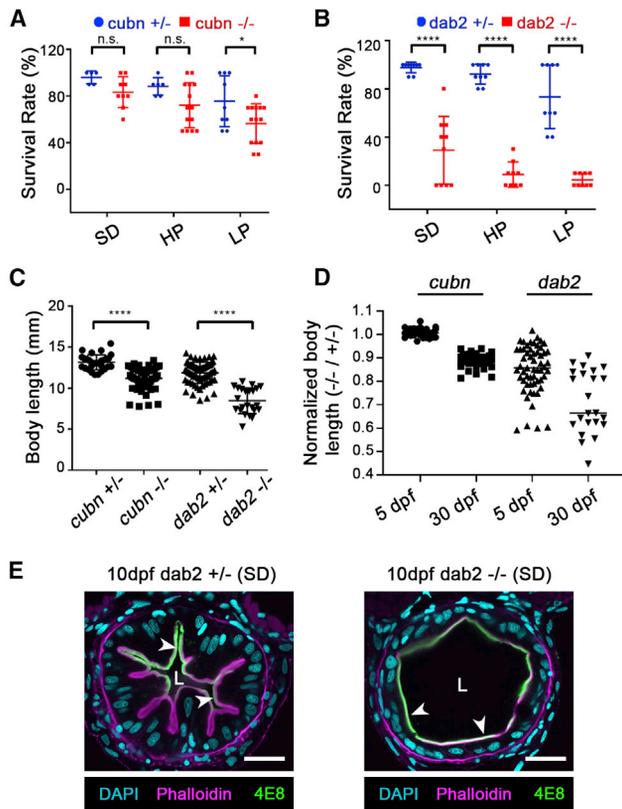
To test whether growth and survival defects that we observed were in part due to essential functions of renally expressed *cubn*, we examined LRE protein uptake, larval survival, and body length in *lrp2a* mutants, which exhibit kidney filtration defects (Anzenberger et al., 2006; Nielsen et al., 2016; Veth et al., 2011). Internalization of Cubn, which lacks transmembrane domain, relies on both Megalin (*Lrp2a*) and *Amn* in this tissue, and loss of *Lrp2a* function also leads to loss of Cubn function in the pronephros (Ahuja et al., 2008; Christensen and Nielsen, 2007). By contrast with *cubn* and *dab2* mutants, we found that *lrp2a* mutants had no mCherry or LY uptake defects, nor survival or growth defects with respect to controls (Figure 6), indicating that *lrp2a* mutants have normal LRE function. These data suggest that the survival defects observed in *cubn* and *dab2* mutants are likely not due to impaired kidney function. Together, these data reveal that protein uptake in LREs is required for normal larval growth and survival in zebrafish. We postulate that the reduced survival of *dab2* with respect to *cubn* mutants is due to the fact that both receptor-mediated and fluid-phase endocytosis of proteins are impaired. Alternatively, it is also possible that other essential soluble factors are also absorbed via LREs.

### Loss of LRE Function Results in Growth Stunting and Intestinal Swelling in Suckling Mice

Classic EM and protein uptake experiments suggest that intestinal LREs present in suckling stage mammals are involved in the uptake of milk components (Gonnella and Neutra, 1984; Kraehenbuhl and Campiche, 1969). Immunohistological data also showed that Cubn and Dab2 are expressed in the ileum of suckling mice and rats (Vázquez-Carretero et al., 2014). We therefore investigated whether LREs are functionally conserved between zebrafish and mice. We first examined mCherry uptake *ex vivo* using ileal segments harvested from P7 suckling mice and observed robust uptake of the protein in cells that closely resemble zebrafish LREs morphologically (Figures 7A and 7B). By contrast, mCherry internalization in duodenum IECs was limited to small vesicular structures (Figure 7G, arrowheads).

#### Figure 4. Dab2 Mediates Fluid-Phase and Receptor-Dependent Endocytosis in LREs

(A) Experimental scheme to mosaically inhibit Dab2 function. A dominant-negative (DN), QUAS:*DN-Dab2:p2A-EGFP*, construct was injected into *Tg(cldn15la:QF2<sup>pd1142</sup>)* embryos to co-express DN-Dab2 and soluble eGFP to mark expressing cells in IECs and LREs. Larvae mosaically expressing DN-Dab2 were then gavaged with fDex or mCherry and imaged 5 h post-gavage.  
(B and C) Live confocal images of fDex or mCherry uptake in 6 dpf *Tg(cldn15la:QF2<sup>pd1142</sup>)* LREs mosaically expressing DN-Dab2. LREs expressing DN-Dab2 (marked with dotted line) showed reduced fDex (n = 6) (B) and mCherry (n = 3) (C) uptake relative to WT neighboring LREs. Scale bar, 20  $\mu$ m.  
(D) Live confocal images of 6 dpf *dab2<sup>pd1162</sup>* *+/+*, *+/-*, *-/-* LREs imaged 5 h after gavage with LY and mCherry. Scale bar, 50  $\mu$ m.  
(E) Internalization profiles of LY (left) and mCherry (right) from 6 dpf *dab2<sup>pd1162</sup>* *+/+* (n = 5), *+/-* (n = 8), *-/-* (n = 6) larvae. *dab2* mutants showed impaired internalization of both protein (mCherry) and fluid-phase (LY) cargos (arrows). Data are means  $\pm$  S.E.M. Images in (C) are digitally stitched. See also Figures S5 and S6.



**Figure 5. Loss of LRE Protein Uptake Impairs Growth and Survival in Larval Zebrafish**

(A and B) Survival rates of *cubn*<sup>pd1169</sup> +/-, -/- (A), and *dab2*<sup>pd1162</sup> +/-, -/- (B) 30 dpf larvae raised under different feeding conditions: non-calorie restricted standard diet (SD), calorie-restricted high-protein diet (HP) and calorie-restricted low-protein diet (LP). n = 5, 9, 6, 14, 9, and 14 (A; left to right), n = 9, 10, 9, 9, 9, and 9 (B; left to right). Data points are the survival rates of each tank subjected to the experiment.

(C) Comparison of body lengths of *cubn*<sup>pd1169</sup> +/- (n = 29), -/- (n = 57), and *dab2*<sup>pd1162</sup> +/- (n = 69), -/- (n = 23) 30 dpf larvae raised under a non-calorie restricted SD. Data points are the body lengths of individual 30 dpf larvae that survived until the end of the feeding experiments.

(D) Quantitation of mutant/heterozygous control body length ratios for *cubn*<sup>pd1169</sup> and *dab2*<sup>pd1162</sup> larvae before onset of feeding (5 dpf) and after being fed with a non-calorie restricted SD (30 dpf). Growth deficits largely occur post-embryonically.

(E) Intestine cross section images of 10 dpf *dab2*<sup>pd1162</sup> +/- or -/- larvae raised under non-calorie restricted SD. Arrowheads point to 4E8 colocalization with phalloidin, indicating no polarization defect in some of the *dab2*<sup>pd1162</sup> -/-. L = lumen. n ≥ 15 animals/genotype. Scale bar, 20 μm. \*p < 0.05, \*\*\*\*p < 0.0001; two-tailed unpaired t tests (A–C). p values, t values and degree of freedom for the statistical tests are provided in the Table S3.

We also found that P7 mouse ileal enterocytes express high levels of Lamp2, labeling large supranuclear compartments similar to those of zebrafish LREs (Figures 7C and 7D). In contrast, IECs in more anterior intestinal segments of both mice and zebrafish showed Lamp2 localization to small puncta (Figures 7E and 7F). These data indicate that LREs are present in the ileum of suckling stage mice and share the same morphology, marker expression, and activity with zebrafish LREs.

Given our finding that Dab2 is essential for LRE function in zebrafish, we next examined its expression in the ileum of pre-

weaning mice. We found that mouse Dab2 is highly enriched in LREs, where it localizes to the subapical domain (Figure 7H). Interestingly, in mammals LREs are present transiently and then replaced at weaning by regular enterocytes (Muncan et al., 2011). We next analyzed previously published ileum transcriptome data (Schjoldager et al., 2008) and found that this developmental transition is associated with a sharp downregulation of ileal Dab2. To define the function of Dab2-expressing LREs in mice, we used a previously developed conditional Dab2 null allele (Dab2<sup>fl/fl</sup>) (Morris et al., 2002) combined with a Villin-Cre (Madison et al., 2002) transgene to generate an intestine-specific conditional knockout (cKO). Similar to our results with zebrafish, we found that loss of Dab2 completely abrogated mCherry uptake in mouse LREs, but did not affect Lamp2 expression or vacuole morphology (Figure 7I). These data indicate that LREs are functionally conserved between zebrafish and mice.

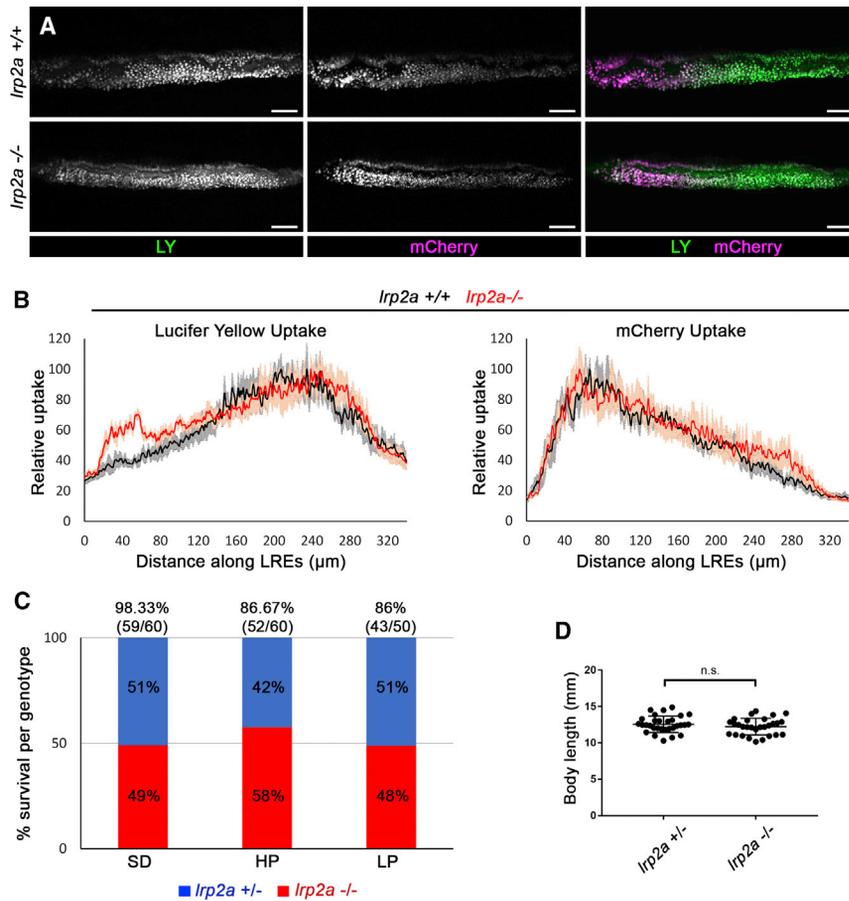
We then monitored the weight and growth of Dab2 cKO mice until weaning (Postnatal day (P) 21–P22) and found that they present significant growth defects in comparison to their sex-matched wild-type (WT) littermates (Figures 7J–7M). Interestingly, a prominent swelling of the GI tract was observed in a large fraction (11 out of 13, p < 0.05) of cKO animals that appeared to result from submucosal edema (Figures 7N–7R). On the other hand, differentiation of goblet cells and crypt morphology were not affected (Figure 7R). Thus, Dab2 has conserved functions in vertebrate LREs. Collectively, our data reveal that LREs are a highly conserved intestinal cell type that plays an essential nutritional role in the early vertebrate gut and are critical for normal development.

## DISCUSSION

Here, we have elucidated the mechanism by which dietary protein is absorbed in the immature gut of zebrafish and suckling mice. We found that a specialized population of intestinal cells that we define as LREs, internalize luminal proteins via fluid-phase and receptor-mediated endocytosis and digest them intracellularly. We show that uptake and internalization of dietary protein is mediated by a receptor complex composed of Cubn, Amn, and the endocytic adaptor Dab2. This molecular machinery is required for LRE function and for growth and survival of larval zebrafish. Importantly, we found that LREs are highly conserved at the morphological and functional level between mouse and zebrafish. Taken together, our results reveal a fundamental and highly conserved cellular mechanism underlying protein absorption in the immature vertebrate gut.

### LREs Are Specialized Intestinal Epithelial Cells that Mediate Dietary Protein Absorption

Histological analyses detected the presence of highly vacuolated enterocytes in the intestine of multiple vertebrate species of mammals and fish (Asari et al., 1987; Trahair and Robinson, 1989; Wallace et al., 2005; Wilson et al., 1991; Zabielski et al., 2008). Tracing of proteins by EM led to the notion that vacuolated enterocytes play a nutritional role by participating in the digestion of milk nutrients via non-selective fluid-phase endocytosis and membrane adsorption (Gonnella and Neutra, 1984; Graney, 1968). However, the validity of these assumptions could not be truly tested because of the lack of understanding of the cellular



**Figure 6. *Irp2a* (megalin) Mutants Do Not Show Defects in LRE Uptake or Survival under Different Feeding Conditions**

(A) Live confocal images showing LY or mCherry uptake in LREs of *Irp2a*<sup>mw1</sup> <sup>+/+</sup>, <sup>-/-</sup> 6 dpf larvae. The larvae were imaged 5 h after gavage. Scale bar, 20  $\mu$ m.

(B) Internalization profiles of LY (left) and mCherry (right) along LREs of 6 dpf *Irp2a*<sup>mw1</sup> <sup>+/+</sup> (n = 5), <sup>-/-</sup> (n = 5). Data are means  $\pm$  S.E.M.

(C) Survival rates of *Irp2a*<sup>mw1</sup> <sup>+/+</sup> and <sup>-/-</sup> 30 dpf larvae raised under different feeding conditions: non-calorie restricted standard diet (SD), calorie-restricted high-protein diet (HP), and calorie-restricted low-protein diet (LP). Larvae from the cross *Irp2a*<sup>mw1</sup> <sup>+/+</sup>  $\times$  *Irp2a*<sup>mw1</sup> <sup>-/-</sup> were used for the experiment. At 30 dpf, the survived larvae were counted and genotyped to determine what percentage of survived larvae are *Irp2a* <sup>+/+</sup> or <sup>-/-</sup>. The numbers above the graph bars indicate the percentage of overall tank survival for each feeding condition.

(D) Comparison of body lengths of *Irp2a*<sup>mw1</sup> <sup>+/+</sup> (n = 30) and <sup>-/-</sup> (n = 29) 30 dpf larvae raised under non-calorie restricted SD. Two-tailed unpaired t test was used for statistical analysis. p values, t values and degree of freedom for the statistical tests are provided in the Table S3.

and molecular mechanisms that control the functions of these cells. Here, we show that, contrary to what was previously proposed, zebrafish LREs specifically internalize proteins from the intestinal lumen via receptor-mediated endocytosis. We found that the multi-ligand receptor Cubn mediates dietary protein internalization (Figures 1I–1M, 3, and S2). Using live imaging in zebrafish, we were able to directly visualize protein uptake, intracellular transport, and degradation in LRE vacuoles, which we found were large lysosomes (Figures 1F–1H). Our studies revealed that LREs have an astounding endocytic and digestive activity that allows them to virtually function as a digestive organ. This striking specialization is reflected at the gene expression level with the upregulation of the lysosomal machinery, particularly of digestive proteases, in LREs with respect to other intestinal cells (Figure S4F). The physiological function of LREs requires that these cells develop a giant lysosomal vacuole, which allows intracellular digestion of internalized proteins. Interestingly, the formation of the lysosomal vacuole was not dependent on the protein internalization activity of these cells (Figures S6E and S6F), indicating the existence of other molecular mechanism that control the specialization of LRE vacuoles.

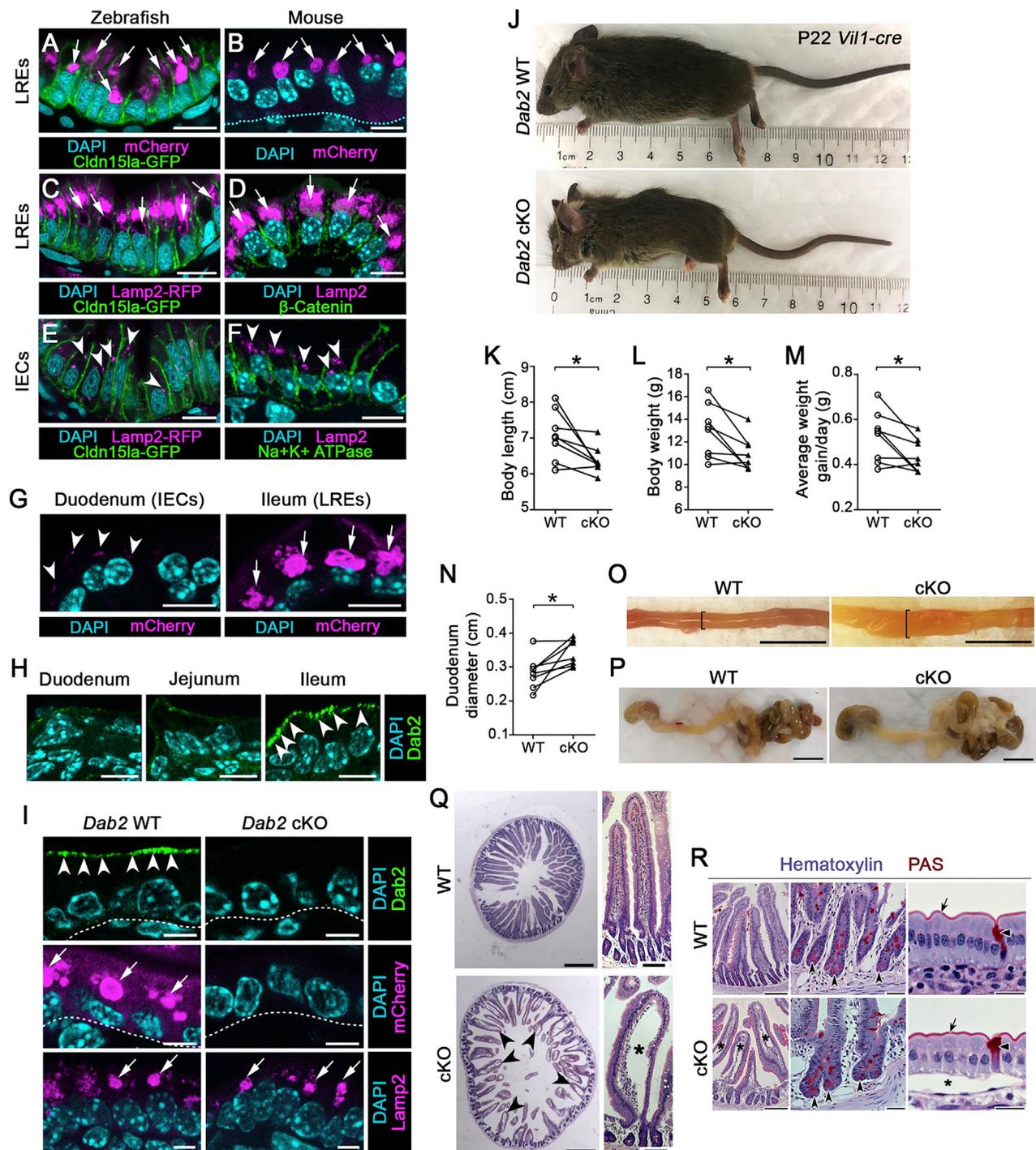
In zebrafish, LREs persist through adulthood (Lickwar et al., 2017; Ng et al., 2005; Wallace et al., 2005); whereas, in mammals they are replaced with adult enterocytes as the intestinal epithelium undergoes major structural and biochemical changes during the suckling to weaning transition (Harper et al., 2011; Muncan et al., 2011). Comparison of our gene expression data with that

of adult zebrafish intestine (Wang et al., 2010) revealed that a number of LRE-enriched genes remain enriched in the homologous region (segment 5) of the adult zebrafish intestine (Figure S7C), which is consistent with a persistence of highly endocytic LREs we observed in adult zebrafish (Figures S7D and S7E). However, future work will be needed to determine whether LREs continue to play a major nutritional role in adult zebrafish.

In contrast to zebrafish, comparison of our gene expression data with that of pre-weaning and post-weaning mouse ileum (Schjoldager et al., 2008) revealed that lysosome and LRE-enriched genes are downregulated in mice after weaning (Figures S7A and S7B). The transition that occurs during weaning in mammals is associated with the change in the expression of the transcriptional repressor *Blimp1* (Harper et al., 2011; Muncan et al., 2011). It will be interesting to investigate whether the differentiation of LREs and the mechanisms controlling the expression of the cellular and molecular machinery that characterizes these cells is conserved between zebrafish and mammals.

### LREs Utilize the Cubn/Amn Scavenger Receptor Complex and the Dab2 Adaptor for Protein Uptake

LREs are remarkably active and effective in retrieving proteins from the intestinal lumen. This is made possible by the activity of the broad-spectrum receptor Cubn. The use of receptor-mediated endocytosis provides a concentrating mechanism that allows efficient protein absorption even when dietary protein is not abundant. While each protein internalized by LREs exhibited distinct uptake profiles (Figures 1J, 1L, and S2A), we also found evidence of competition between protein cargoes (Figures 1M and S2B–S2D), suggesting that Cubn recognizes a



**Figure 7. Loss of LRE Function Results in Growth Stunting and Intestinal Swelling in Suckling Mice**

(A and B) Confocal images of LREs of *TgBAC(cldn15la-GFP<sup>pd1034</sup>)* zebrafish larva (A) and of P7 neonatal mouse (B) after mCherry uptake. Arrows point to lysosomal vacuoles filled with mCherry. Dashed line in (B) indicates basement membrane. Scale bar, 10  $\mu$ m.

(C–F) Confocal images of LREs (C and D) and IECs (E and F) of *TgBAC(cldn15la-GFP<sup>pd1034</sup>)*; *TgBAC(lamp2-RFP<sup>pd1044</sup>)* zebrafish larva and P7 neonatal mouse intestine stained with LAMP2 antibody, showing the presence of lysosomal vacuoles (arrows) in LREs (C and D) and lysosomes (arrowheads) in IECs (E and F). Scale bar, 10  $\mu$ m.

(G) Confocal images of duodenum and ileum of P6 neonatal mouse after mCherry uptake. Arrowheads point to small vesicular structures with mCherry in IECs and arrows point to lysosomal vacuoles filled with mCherry in LREs. Scale bar, 10  $\mu$ m.

(H) DAB2 expression in the duodenum, jejunum, and ileum of P3 WT neonatal mouse. DAB2 expression is enriched in the ileum (arrowheads), the segment harboring LREs. Scale bar, 10  $\mu$ m.

(legend continued on next page)

general protein feature with variable affinity. Alternatively, each protein might have a different number of epitopes recognized by Cubn.

The survival of *dab2* zebrafish mutants was more severely compromised compared to that of *cubn* mutants in all feeding regimens (Figure 5B), likely due to the fact that Dab2 is involved in both fluid-phase and receptor-mediated endocytosis (Figure 4). This suggests that sufficient absorption of dietary proteins needs both fluid-phase and receptor-mediated endocytosis. Alternatively, some of the non-protein soluble cargoes, internalized via the fluid-phase endocytosis in LREs, might be essential for larval survival and growth.

Replacement of LREs by regular enterocytes in mice correlates with weaning and coincides with a significant reduction in the expression of *Dab2* (Schjoldager et al., 2008) (Figures S7A and S7B), suggesting Dab2 function determines the endocytic capacity of intestinal cells. However, ectopic expression of Dab2 in enterocytes did not enhance their protein uptake capacity (data not shown). Identification of co-regulated genes that are differentially expressed in LREs versus other IECs may reveal the core genetic program driving LRE physiology.

### Systemic Roles of LREs in Health and Disease

We found that LREs are remarkably conserved between zebrafish and mice. At the functional level, this is reflected in their core nutritional function, which allows efficient protein absorption via a conserved molecular machinery. The conserved function of LREs may also extend to their role in trans-cellular transport, which allows the passage of intact proteins and other soluble cargoes across the intestinal barrier (Figure S3). In zebrafish, while most cargoes internalized by LREs are destined to lysosomal vacuole where they are digested, some of the cargoes undergo trans-cellular transport across LREs and accumulate in extra-intestinal tissues over time (Figure S3). Similarly, in neonatal mammals, transport of antibodies and luminal antigens across intestinal epithelium is highly active (Buchanan et al., 2012; Turfkruyer et al., 2016). This phenomenon has been attributed to the presence of an immature intestinal permeability barrier. However, our results indicate that in zebrafish, LREs, and their Cubn/Amn/Dab2 machinery provide a major route for trans-epithelial transport (Figures S3C and S3D), suggesting that LREs are also likely responsible for the high transcellular permeability of the mammalian neonatal intestine. This conserved activity may provide a high-capacity route for trans-cellular transports of intact proteins like antibodies and other soluble antigens including bio-active microbial products (Hill

et al., 2016). We speculate that LREs may thus facilitate passive immunity in mammals as well as the development of the innate immune system, tolerance to food and microbial antigens, and host-microbe communication in vertebrates.

Interestingly, we found that both Tfeb and Slc38a9, which are components of the mTORC1 pathway (Rebsamen et al., 2015), are highly upregulated in LREs compared to IECs (not shown). While this may simply reflect the abundance of lysosomes in LREs, it is tempting to speculate that LREs may sense the availability of dietary nutrients. Since LREs internalize dietary proteins and digest them intracellularly, the level of amino acids within the lysosomal vacuole should reflect dietary nutrient availability.

Our mouse data show that loss of *Dab2* in the intestine leads to growth stunting and intestinal swelling, which is reminiscent of kwashiorkor. Kwashiorkor is a disease caused by severe protein malnutrition and is characterized by the presence of edema, dermatosis, and diarrhea (Golden, 1998; Williams et al., 2003). The origin of kwashiorkor has been debated and remains poorly understood, and potential causes of the disease include low plasma albumin, low protein uptake, and microbiota disturbances (Coulthard, 2015; Smith et al., 2013b). While our data supports low protein uptake as a leading cause of kwashiorkor, it is also possible that insufficient LRE protein uptake in *Dab2* cKO mice leads to an alteration in their gut microbiota, which in turn may cause intestinal swelling. Indeed, earlier gnotobiotic experiments in zebrafish suggest that LRE function is regulated by host-microbe interactions (Bates et al., 2006; Rawls et al., 2004). Therefore, it is possible that a dysbiosis of environmental origin may lead to a loss of LRE activity that would in turn cause protein malabsorption. Further investigation of this mouse model may provide insights into the effects of perinatal protein malnutrition in humans.

In conclusion, our work shows an important physiological role for LREs that is remarkably conserved at the molecular and functional level between zebrafish and mice. Future research of the biology of LREs has the potential to illuminate how vertebrate animals interact with the environment as they develop and mature.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY

(I) mCherry uptake in the ileum of P6 *Dab2* WT and *Dab2* cKO (*Vil1-Cre;Dab2<sup>tm1Cpr</sup>/fl/fl*) mice. While DAB2 expression (top images; arrowheads) and mCherry protein uptake (middle images; arrows) is abolished in *Dab2* cKO mouse ileal LREs, LAMP2 expression is not (bottom images; arrows). Scale bar, 5  $\mu$ m.

(J) Images of P22 *Dab2* WT and *Dab2* cKO mice.

(K–N) Quantitation of body length (K), weight (L), average weight gain/day (M), and duodenum diameter (N) of P22 *Dab2* WT and cKO mice.

(O) Images of dissected duodenum of P22 *Dab2* WT and cKO mice. Brackets show prominent swelling of the GI tract in *Dab2* cKO mice. Scale bar, 1 cm.

(P) Images of the dissected stomach and intestinal tract of WT and *Dab2* cKO mice harvested at P22. Scale bar, 1 cm.

(Q) Images of H&E stained sections of the duodenum of P22 *Dab2* WT and cKO mice, which exhibit severe distended villi (arrowheads and asterisk) indicative of submucosal edema. Scale bar, 500  $\mu$ m (transverse cross sections), 100  $\mu$ m (longitudinal cross sections).

(R) Hematoxylin and periodic acid–Schiff (PAS) staining of duodenal sections of WT and *Dab2* cKO P22 mice. Concave arrowheads point to Paneth cells within crypts; flat arrowheads show goblet cells; arrows mark apical membrane; asterisks indicate edema. Scale bar, 200  $\mu$ m (left), 40  $\mu$ m (middle), and 20  $\mu$ m (right). In (K–N), data points are littermate averages for each genotype and littermate averages of WT and cKO from the same litter are paired with lines. Measurements of individual mice from 8 different litters graphed in (K–N) are provided in the Table S2. \* $p < 0.05$ ; Two-tailed paired t tests (K–N). p values, t values, and degree of freedom for the statistical tests are provided in Table S3.

## ● EXPERIMENTAL MODELS AND SUBJECT DETAILS

- Fish
- Mice

## ● METHOD DETAILS

- Genome Editing
- Transgenesis
- Soluble Fluorescent Protein Production
- Gavage
- Quantification of Relative Uptake by LREs
- Cell Isolation and RNA Sequencing
- Analysis of Gene Expression Data
- RNA Isolation and Reverse Transcription PCR (RT-PCR)
- Quantitative PCR
- *In Situ* Hybridization
- Microscopy
- Electron Microscopy
- Zebrafish Growth and Survival under Controlled Feeding Conditions
- Immunofluorescence and Histology
- *Ex Vivo* MCherry Uptake in Mouse Intestinal Tissue

## ● QUANTIFICATION AND STATISTICAL ANALYSIS

## ● DATA AND CODE AVAILABILITY

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.devcel.2019.08.001>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, M.B. and J.P.; Methodology, M.B. and J.P.; Investigation, J.P., D.S.L., K.D.S., C.L.B., O.E., and J.B.; Formal Analysis, C.R.L.; Resources, R.B., S.A.W., J.F.R., C.E., and T.L.; Writing – Original Draft, M.B. and J.P.; Writing – Review & Editing, M.B., J.P., D.S.L., T.L., and C.E.; Supervision, M.B.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Rabbit polyclonal anti-Rab5	GeneTex	Cat# GTX13253; RRID: AB_383116
Rabbit monoclonal anti-Rab7 (D95F2)	Cell Signaling	Cat# 9367; RRID: AB_1904103
Mouse monoclonal anti-Disabled-2/p96 (Clone 52/p96)	BD Biosciences	Cat# 610464; RRID: AB_397837
Mouse monoclonal anti-LAMP-2	DSHB	Cat# ABL-93; RRID: AB_2134767
Chicken monoclonal anti-ATPase, (Na <sup>+</sup> ), K <sup>(+)</sup> ) alpha subunit	DSHB	Cat# a5; RRID: AB_2166869
Monoclonal anti- $\beta$ -catenin (Clone 15B8)	Sigma-Aldrich	Cat# C7207; RRID: AB_476865
Anti-Zebrafish Gut Absorptive Cell Epitopes antibody [FIS 4E8/1]	Abcam	Cat# ab73643; RRID: AB_1269042D
Alexa Fluor <sup>TM</sup> 568 Phalloidin	ThermoFisher	Cat# A12380
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Lucifer Yellow CH, Lithium Salt	ThermoFisher	Cat# L453
Transferrin From Human Serum, Alexa Fluor <sup>TM</sup> 488 conjugate	ThermoFisher	Cat# T13342
Dextran, Alexa Fluor <sup>TM</sup> 568; 10,000 MW	ThermoFisher	Cat# D22912
Lysotracker <sup>TM</sup> Red DND-99	ThermoFisher	Cat# L7528
BODIPY <sup>TM</sup> FL C16	ThermoFisher	Cat# D3821
SensiFAST <sup>TM</sup> SYBR <sup>®</sup> No-ROX Kit	Bioline	Cat# BIO-98005
Trypsin-EDTA	ThermoFisher	Cat# 25200056
Collagenase	Sigma-Aldrich	Cat# C2674
Propidium Iodide	ThermoFisher	Cat# P3566
BM-Purple	Roche	Cat# 11442074001
<b>Critical Commercial Assays</b>		
RNeasy Plus Micro Kit	Qiagen	Cat# 74034
<b>Deposited Data</b>		
Raw and analyzed data for RNA-seq experiment	this paper	GEO: GSE124970
<b>Experimental Models: Organisms/Strains</b>		
Zebrafish / <i>cubn</i> <sup>pd1169</sup>	this work	N/A
Zebrafish / <i>dab2</i> <sup>pd1162</sup>	this work	N/A
Zebrafish / <i>amn</i> <sup>pd1189</sup>	this work	N/A
Zebrafish / <i>Tg(cldn15la:QF2)</i> <sup>pd1142</sup>	this work	N/A
Zebrafish / <i>Tg(QUAS:GFP-Rab32a)</i> <sup>pd1201</sup>	this work	N/A
Zebrafish / <i>Tg(QUAS:mCherry-snx27a)</i> <sup>pd1177</sup>	this work	N/A
Zebrafish / <i>Tg(hsp:Venus-FYVE)</i> <sup>pd1046</sup>	(Ellis et al., 2013)	N/A
Zebrafish / <i>Tg(hsp:laGFP)</i> <sup>pd1205</sup>	this work	N/A
Zebrafish / <i>Tg(2.3k ifabp:sNgly-RFP)</i> <sup>pd1206</sup>	this work	N/A
Zebrafish / <i>lrp2a</i> <sup>mw1</sup>	(Veth et al., 2011)	N/A
Zebrafish / <i>TgBAC(cldn15la-GFP)</i> <sup>pd1034</sup>	(Alvers et al., 2014)	N/A
Zebrafish / <i>TgBAC(lamp2-RFP)</i> <sup>pd1044</sup>	(Rodríguez-Fraticelli et al., 2015)	N/A
Zebrafish / <i>TgBAC(anxa2b-RFP)</i> <sup>pd1113</sup>	(Marjoram et al., 2015)	N/A
Mouse / Villin-Cre	(el Marjou et al., 2004)	N/A
Mouse / Villin-CreER	(el Marjou et al., 2004)	N/A
Mouse / <i>Dab2</i> <sup>fl/fl</sup> (B6; 129S4- <i>Dab2</i> <sup>tm1Cpr/J</sup> )	Jackson Laboratory (Morris et al., 2002)	Cat# JAX:022837; RRID:IMSR_JAX:022837

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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Oligonucleotides</b>		
Primers for cloning pME-DN-Dab2: forward: 5' CCACTCACCATGACTGCACCCGTGACCAG 3'	this work	N/A
Primers for cloning pME-DN-Dab2: reverse: 5' CTTTGTCCAGCCTCCTGCTC 3'	this work	N/A
Genotyping primers for <i>cubn</i> <sup>pd1169</sup> : forward: 5' ACTCTGTTACCTGCAGTGC 3'	this work	N/A
Genotyping primers for <i>cubn</i> <sup>pd1169</sup> : reverse (DCAPS): 5' TGACATCCGAGTGGAGTTCCTGCCAAGAC 3'	this work	N/A
Genotyping primers for <i>dab2</i> <sup>pd1162</sup> : forward: 5' ACAGACGAGTTCCTCTTGGC 3'	this work	N/A
Genotyping primers for <i>dab2</i> <sup>pd1162</sup> : reverse, 5' CCGCTTCTTGCACTGCTAGA	this work	N/A
Genotyping primers for <i>amn</i> <sup>pd1189</sup> : forward: 5' GGATGAGCTTGCTGTGGAT 3'	this work	N/A
Genotyping primers for <i>amn</i> <sup>pd1189</sup> : reverse, 5' GAATGAGGTGGCGTCTCGAA 3'	this work	N/A
Primers for generating in situ probes for <i>dab2</i> and <i>amn</i> , see <a href="#">Table S4</a>	this work	N/A
<b>Recombinant DNA</b>		
p5E-QUAS	(Subedi et al., 2014)	N/A
p5E-cldn15la	(Murdoch et al., 2019)	N/A
pME-QF2	(Subedi et al., 2014)	N/A
pME-DN-Dab2	this work	N/A
pRSETB-mCherry	(Sarabipour et al., 2014)	N/A
pRSETB-mTurquoise	(Sarabipour et al., 2014)	N/A
pGEMTeasy-lrp2a	(Anzenberger et al., 2006)	N/A
pCR TOPO-cubilin	(Anzenberger et al., 2006)	N/A
<b>Software and Algorithms</b>		
DAVID Bioinformatics Resources 6.8	(Huang da et al., 2009a, 2009b)	<a href="https://david.ncifcrf.gov/">https://david.ncifcrf.gov/</a>
DESeq2	(Anders et al., 2015; Love et al., 2014)	<a href="https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/deseq2/deseq2/2.11.39">https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/deseq2/deseq2/2.11.39</a>
htseq-count	(Kim et al., 2015)	<a href="https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/lparsons/htseq_count/htseq_count/0.6.1galaxy3">https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/lparsons/htseq_count/htseq_count/0.6.1galaxy3</a>
HISAT2	(Kim et al., 2015)	<a href="https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/hisat2/hisat2/2.0.5.2">https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/hisat2/hisat2/2.0.5.2</a>
Graphpad Prism	GraphPad Software, La Jolla, CA, USA	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a> ; RRID: SCR_002798

**LEAD CONTACT AND MATERIALS AVAILABILITY**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michel Bagnat ([michel.bagnat@duke.edu](mailto:michel.bagnat@duke.edu)). Zebrafish lines, plasmids, and other reagents generated in the study will be available upon request.

## EXPERIMENTAL MODELS AND SUBJECT DETAILS

### Fish

All fish were used in accordance with the Duke University Institutional Animal Care and Use Committee (IACUC) guidelines. Zebrafish (*Danio rerio*) stocks were maintained at 28°C in a recirculating system and bred by putting a male and a female zebrafish in a mating tank and controlling the day-night cycle (Westerfield, 2000). Genotypes were determined by fin clipping. Male and female breeders from 3-9 months of age were used to generate fish for all experiments. 5-30 dpf zebrafish larvae from the Ekkwill (EK) or AB/TL background were used in this study. No test on the influence of sex were performed as sex determination in zebrafish occurs in late juvenile stage, well after our experimental window. Strains generated for this study: *cubn*<sup>pd1169</sup>, *dab2*<sup>pd1162</sup>, *amn*<sup>pd1189</sup>, *Tg(cldn15la:QF2)*<sup>pd1142</sup>, *Tg(QUAS:GFP-Rab32a)*<sup>pd1201</sup>, *Tg(QUAS:mCherry-snx27a)*<sup>pd1177</sup>, *Tg(hsp:laGFP)*<sup>pd1205</sup>, *Tg(2.3k ifabp:sNgly-RFP)*<sup>pd1206</sup>. Previously published strains: *Irp2a*<sup>mW1</sup> (Veth et al., 2011), *TgBAC(cldn15la-GFP)*<sup>pd1034</sup> (Alvers et al., 2014), *TgBAC(lamp2-RFP)*<sup>pd1044</sup> (Rodríguez-Fraticelli et al., 2015), *TgBAC(anxa2b-RFP)*<sup>pd1113</sup> (Marjoram et al., 2015), *Tg(hsp:Venus-FYVE)*<sup>pd1046</sup> (Ellis et al., 2013).

### Mice

All mice were used in accordance with the Duke University Institutional Animal Care and Use Committee (IACUC) and the Duke Division of Laboratory Animal Resources (DLAR) oversight. The *Dab2* floxed (Morris et al., 2002) (B6;129S4-*Dab2*<sup>tm1Cpr/J</sup>; Cat# JAX:022837; RRID: IMSR\_JAX:022837) line was obtained through Jackson Laboratory. The *Dab2* floxed mice were crossed to villin-Cre (el Marjou et al., 2004) mice to generate intestinal epithelial-specific *Dab2* conditional knockout (cKO) mice. Mice were genotyped by PCR, and both males and females were analyzed. Age and sex-matched pairs were selected for the paired comparison of WT and cKO mice as shown in Table S2. No test was performed to determine the influence of sex as no obvious differences were found. Mice were maintained in a barrier facility with 12-hour light/dark cycles. Mice had access to water and food *ad libitum*. The weight of *Dab2* WT and *Dab2* cKO mice were measured every three days from P0 to P22. At P22, the weight and length of mice were measured, and the intestine was dissected after sacrifice for further analysis. Intestinal length was measured, as was the diameter of the duodenum.

## METHOD DETAILS

### Genome Editing

Mutant lines were generated using CRISPR/Cas9. Guide RNA (gRNA) target sites were identified using CRISPRscan (Moreno-Mateos et al., 2015) and gRNAs were synthesized using the oligo-based method (Yin et al., 2015). *cubn*<sup>pd1169</sup> mutants were generated using a gRNA targeting exon 5, *dab2*<sup>pd1162</sup> mutants at exon 5, and *amn*<sup>pd1189</sup> mutants at exon 1. gRNA target sequences were: *cubn*- 5' TGTGAATGAGTGTGTCAGGTGT 3', *dab2*-5' TGATGTTCAAGATGCAAGAG 3', *amn*- 5' GGGTTTACTGTGCGAGAAGG 3'. Zebrafish embryos were injected at the one cell stage with 150 pg/nl of Cas9 mRNA and 50 pg/nl of gRNA. Genotyping for *cubn* was performed using primers: forward, 5' ACTCTGTTACCTGCAGTGC 3'; (DCAPS primer (Neff et al., 1998)) reverse, 5' TGACATCCGAGTGGAGTTCCTGCCAAGAC 3'. PCR products were then digested with *HinfI* enzyme for genotyping. Genotyping for *dab2* was performed using primers: forward, 5' ACAGACGAGTTCCTCTTGGC 3'; reverse, 5' CCGCTTCTTGCACTGCTAGA 3'. Genotyping for *amn* was performed using primers: forward, 5' GGATGAGCTTGCTGTGGAT 3'; reverse, 5' GAATGAGGTGGCGTCTCGAA 3'.

### Transgenesis

All constructs for transgenic fish were generated using the Tol2kit gateway cloning system using the p5E-QF2, p5E-cldn15la, pME-QF2, pME-DN-Dab2, p3E-polyA, p3E-p2A-eGFP, and pDestTol2CG2 vectors (Kwan et al., 2007; Ellis et al., 2013). The p5E-QUAS and pME-QF2 vectors were gifts from Marnie Halpern (Subedi et al., 2014). The p5E-cldn15la vector was a gift from John Rawls (Murdoch et al., 2019).

To generate pME-DN-Dab2, we amplified the PTB domain of *Dab2*, which acts as a dominant negative (Mishra et al., 2002). DN-Dab2 was amplified using the following primers: forward, 5' CCACTCACCATGACTGCACCCGTGACCAG 3'; reverse, 5' CTTTGTCCAGCCTCCTGCTC 3'.

### Soluble Fluorescent Protein Production

pRSET-mCherry and pRSETB-mTurquoise vectors were gifts from Kalina Hristova. MCherry and mTurquoise soluble fluorescent proteins were produced by uninduced expression in the BL21-Gold (DE3) strain of *Escherichia coli*. The fluorescent proteins were purified from the pellet of large bacterial culture using the His tag (Sarabipour et al., 2014).

### Gavage

5-6 dpf larvae were gavaged with 4 nl of 1.25 mg/ml mixture of lucifer yellow (Thermo Fisher Scientific), Alexa Fluor 488 conjugated transferrin (Thermo Fisher Scientific), Alexa Fluor 568 dextran (Thermo Fisher Scientific), LysoTracker (Thermo Fisher Scientific), mCherry or mTurquoise for different gavage experiments using a previously described gavage technique (Cocchiari and Rawls, 2013). In brief, the gavage technique is performed with a microforged needle which goes through the zebrafish larvae mouth and

esophagus to directly inject the injection mixture into the gut lumen of zebrafish larvae. For protein competition assays (Figures 1M and S2B–S2D), higher concentrations of proteins were used (Indicated in the graphs). Gavaged larvae were imaged 4–5 hours after gavage using a FluoView FV3000 (Olympus) confocal microscope. For lipid and protein uptake assays (Figures 1D and S6), 5  $\mu$ l of 10 mg/ml BODIPY<sup>TM</sup> FL C16 (ThermoFisher Scientific) was air dried and dissolved in 5  $\mu$ l of 100% EtOH. Then 50  $\mu$ l of 5% egg yolk solution was added to make 1 mg/ml BODIPY<sup>TM</sup> FL C16 solution. 3  $\mu$ l of 1 mg/ml BODIPY<sup>TM</sup> FL C16 solution and 1  $\mu$ l 5 mg/ml mCherry solution were mixed to make the final concentration of BODIPY<sup>TM</sup> FL C16 and mCherry in the mixture to be 0.75 mg/ml and 1.25 mg/ml, respectively. Larvae were gavaged with 4 nl of the mixture and imaged after 1 hour using a FluoView FV3000 (Olympus) confocal microscope.

### Quantification of Relative Uptake by LREs

To quantify the relative amount of uptake along the length of LREs, images of LREs were analyzed with ImageJ software (NIH) using the “Plot Profile” function to obtain gray value along the length (pixel) of the image. Then, pixels were converted to  $\mu$ m and the gray values were used to calculate relative uptake of fluorescent cargoes. To visualize gavaged cargoes accumulation inside the lumen (Figures S1G, S1H, S1J, and S1K), a line was drawn on optical sections of LRE confocal images and a line scan was performed using ImageJ. For kinetic analysis of mCherry or mTurquoise uptake over time (Figure 1G), 5 dpf larvae were gavaged with 4 nl of 1.25 mg/ml mCherry or mTurquoise and then fixed in 4% paraformaldehyde (PFA) at the indicated timepoints. Whole larvae were mounted on glass bottom dishes and LREs were imaged using confocal microscopy. Mean pixel intensity measurements of the vacuole (delimited by GFP-Rab32) and the whole cell were taken with ImageJ, and ratios of the two values for individual cells were plotted and analyzed using GraphPad Prism (GraphPad Software). For kinetic analysis of mCherry or mTurquoise degradation (Figure 1H), 5 dpf larvae were gavaged with 4 nl of 1.25 mg/ml mCherry or 5 mg/ml mTurquoise and incubated at 28°C for one hour to allow protein uptake by LREs. Then, the larvae were gavaged again with PBS to flush the remaining luminal protein pool. Then, larvae were fixed in 4% PFA at the indicated timepoints and LREs were imaged using confocal microscopy. Mean pixel intensity measurements of anterior LREs were taken with ImageJ and used to calculate relative fluorescence intensity. The values were plotted and analyzed using GraphPad Prism.

### Cell Isolation and RNA Sequencing

For isolating IECs and LREs, 5 dpf *TgBAC(cldn15la-GFP)<sup>pd1034</sup>* fish were gavaged with 1–2 nl of 2.5 mg/ml Alexa Fluor 568-dextran, incubated at 28°C for 24 hours. Then, larvae were dissociated to generate single-cell suspensions for FACS by incubating in 0.25% trypsin-EDTA (Life Technologies) and 1% collagenase (Sigma-Aldrich) at 28°C for approximately 1.5 hr. Dissociations were encouraged by pipetting every 15 min. Cells were then washed with PBS plus 5% fetal calf serum (FCS) and passed through a 30  $\mu$ m CellTrics filter (Sysmex). Cell suspensions were stained with 7-AAD (Sigma) and sorted on a MoFlo Astrios EQ cell sorter (Becton Coulter) at the Flow Cytometry Shared Resource Center (Duke University). Cells were collected in Buffer RLT Plus (Qiagen) and total RNA was prepared from each population using RNeasy Plus Micro Kit (Qiagen). RNA samples were evaluated for concentration by Qubit (Thermo Fisher Scientific) and for integrity using an Agilent 2100 Bioanalyzer. Samples with an RNA integrity number (RIN) >7.0 were used for RNA sequencing (RNA-seq).

Clontech Ultra low libraries were prepared in triplicate and sequenced using the Illumina HiSeq 2000 50 bp single-end read platform. Sequencing data were uploaded to the Galaxy Web platform, and we used the public server at <https://usegalaxy.org> for analysis (Afgan et al., 2018). Reads were mapped to the GRCz10 (danRer10) zebrafish genome using HISAT2 (Kim et al., 2015), and gene counts were analyzed using HTseq (Anders et al., 2015). HTseq-counts were input into DESeq2 (Love et al., 2014) to calculate differential expression for cell populations (Wopat et al., 2018). Genes were considered to be enriched in a population if their expression had a  $\log_2$ (fold change)  $\geq 2$  in comparison with the other population, with an adjusted p value of <0.05. Principal-component analysis was performed in R using the DESeq2 package. All KEGG pathway and GO term analysis was performed using David Bioinformatics Resources 6.8 (Huang da et al., 2009a, 2009b; Kanehisa and Goto, 2000). Heatmaps were generated using the gplots package in R.

### Analysis of Gene Expression Data

To process mouse ileum time course data (Figures S7A and S7B), normalized expression data from each replicate of time course data from GEO accession GSE8065 (Schjoldager et al., 2008) was clustered using LRE-enriched mouse orthologs. Data were gene median centered and hierarchically clustered using complete linkage with uncentered correlation by Cluster 3.0. GEO2R (<https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE8065>) was used to generate  $\log_2$  fold change data between day 32 and day 7 timepoints using all replicates with the default parameters. LRE-enriched mouse orthologs that were identified by clustering to be down regulated post-weaning were used as input for Enrichr 3.0 (<https://amp.pharm.mssm.edu/Enrichr/>). Lysosome genes were extracted from KEGG ([https://www.genome.jp/dbget-bin/www\\_bget?pathway+mmu04142](https://www.genome.jp/dbget-bin/www_bget?pathway+mmu04142)). Orthology definitions between zebrafish and mouse were downloaded from Ensembl BioMart Genes 96.

To compare the gene expression data of larval zebrafish intestine to that of adult zebrafish intestine (Wang et al., 2010) (Figure S7C), the Z-score values of the adult zebrafish segment 5 (ileum-like) relative to all remaining segments were median centered for each gene and the details of the data processing are described in (Lickwar et al., 2017).

### RNA Isolation and Reverse Transcription PCR (RT-PCR)

RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using first Strand cDNA Synthesis Kit (Roche). To generate cDNA for RT-PCR for *cubn*, *Cubn\_R* primer, 5' GGCAGACATAACCCAGTGGCT 3', was used for reverse transcription cDNA synthesis. To generate cDNA for RT-PCR for *amn* and *dab2*, poly dT primer was used for reverse transcription cDNA synthesis. Primers used for RT-PCR include: *cubn\_RT\_F*, 5' CAGCAGTAACCCATGCCAGA 3'; *cubn\_RT\_R*, 5' GGAACCCAGCGTGTGAAAC 3'; *dab2\_RT\_F*, 5' ACAGACGAGTTCCTCTTGGC 3'; *dab2\_RT\_R*, 5' AGCCA AATGCCCGTTATCA 3'; *amn\_RT\_F*, 5' GGATGAGCTTGCCTGTGGAT 3'; *amn\_RT\_R*, 5' GAATGAGGTGGCGTCTCGAA 3'; *rpl13a\_F*, 5' TCTGGAGGACTGTAAGAGGTATGC 3'; *rpl13a\_R*, 5' AGACGCACAATCTTGAGAGCAG 3'. *rpl13a* was used as standard for all RT-PCR experiments.

### Quantitative PCR

Quantitative PCR was performed using a Bio-Rad CFX96 Real-Time System C1000 Thermocycler and SensiFAST™ SYBR No-ROX kit (Bioline). Reactions were performed in duplicate. Primers used include: *rpl13a\_F*, TCTGGAGGACTGTAAGAGGTATGC; *rpl13a\_R*, 5' AGACGCACAATCTTGAGAGCAG 3'; *ifabp\_F*, 5' CCACTGTCAGGATCACACAAC 3'; *ifabp\_R*, 5' AGCCAGTTTCCTTTTCACCATG 3'; *mtp\_F*, 5' CGGATCGTCTCTGCTTATTC 3'; *mtp\_R*, 5' GTTGTGCTCTGACCGTAAATG 3'; *lamp2\_F*, 5' GTCTGCTCAGATAAAC CATCCAC 3'; *lamp2\_R*, 5' TCATCATATCATCAGCATGAAC 3'; *pllp\_F*, 5' GTGGGTATTACCAGGGTTCATC 3'; *pllp\_R*, 5' GGCTGTGTATAACAGGGTCTCC 3'. Expression levels were normalized to *rpl13a* for each cDNA set.

### In Situ Hybridization

In situ probes for *lrp2a* and *cubn* were generated using the plasmids generously shared by Salim Abdelilah-Seyfried (Anzenberger et al., 2006). pGEMteasy-*lrp2a* and pTOPO-*cubn* plasmids were linearized using *SpeI* and *BamHI*, respectively, and subjected to *in vitro* transcription using T7 RNA polymerase (NEB) and T3 RNA polymerase (Roche), respectively. To make *in situ* probes for *dab2* and *amn*, *dab2* and *amn* were amplified from cDNA using the following primers: *dab2\_antisense probe\_F*, 5' CCCAGTGG TCAAAGTGCCT 3'; *dab2\_antisense probe\_R*, 5' ATAGCTAATACGACTCACTATAGGGAGCTACTTCACTGTGCAGCT 3'; *dab2\_sense probe\_F*, 5' ATAGCTAATACGACTCACTATAGGGGAAAGAAGAGCCCCCAGTGG 3'; *dab2\_sense probe\_R*, 5' AGCTACTT CACTGTGCAGCT 3'; *amn\_antisense probe\_F*, 5' GCTGCTGGGAGTGATTCCTT 3'; *amn\_antisense probe\_R*, 5' ATAGCTAATAC GACTCACTATAGGGTGAAGCTTGTGAGAACTCCCA 3'; *amn\_sense probe\_F*, 5' ATAGCTAATACGACTCACTATAGGGGGCTG CTGGGAGTGATTCCTT 3'; *amn\_sense probe\_R*, 5' TGAAGCTTGTGAGAACTCCCA 3'. Using the T7 promoter sequence included in the reverse primers for antisense probes and in the forward primers for sense probes, the PCR products were subjected to *in vitro* transcription using T7 RNA polymerase (NEB). All *in vitro* transcription reactions were performed with DIG RNA Labeling Mix (Roche) to make digoxigenin-labeled RNA. In situ hybridization for *lrp2a*, *cubn*, *dab2* and *amn* was performed as described previously (Navis et al., 2013). Briefly, 5–6 dpf EK embryos were fixed in 4% PFA, dehydrated in 100% MeOH, and rehydrated. Then samples were digested with proteinase K, hybridized with *in situ* probes, washed multiple times, incubated with secondary antibody, and stained using BM-Purple (Roche). Images were acquired on a Discovery V20 stereoscope (Zeiss).

### Microscopy

Whole-mount confocal live imaging was performed on a FluoView FV3000 (Olympus) confocal microscope equipped with 30x/1.05 silicone oil objective (Olympus) and FluoView software (Olympus). Fish were mounted onto glass-bottom dishes in a 1.2% agarose mixture of egg water and 1x tricaine. Z-step size for z-stacks images was 1.5 μm. Digital stitching of confocal images (Figures 1D, 1I, 4D, S3A–S3D, and S6A–S6D) was done in FluoView software.

### Electron Microscopy

Zebrafish larvae were fixed in 0.1M sodium cacodylate, and 2.5% glutaraldehyde (GA). Specimen preparation and staining was done as previously described (Garcia et al., 2017). Briefly, after fixation, samples were stained with osmium tetroxide and uranyl acetate. Then samples were then dehydrated in ethanol solutions of increasing concentration and embedded in resin blocks overnight and then embedded and cured in 60 degrees oven for 48 hr. Then thin sections were cut and post-stained with lead citrate and uranyl acetate and placed on copper grids for imaging.

### Zebrafish Growth and Survival under Controlled Feeding Conditions

For larval feeding experiments, 10 larvae/3L tank were placed on our recirculating Aquatic Habitats system at 6 dpf. For non-calorie restricted standard feeding condition, each tank was fed with larval AP100 (Zeigler; twice daily) and GM75 (Skretting; once daily) from 5 dpf to 12 dpf and with artemia (twice daily) and GM75 (once daily) from 13 dpf to 30 dpf. For calorie-restricted high-protein (HP) or low-protein (LP) diet feeding conditions, each tank was fed with 10 mg of HP or LP once a day. HP and LP custom diets were formulated to have 50.10% or 25.05% protein contents, respectively. Diets are formulated as described before (Smith et al., 2013a). Briefly, each diet was produced using chemically defined ingredients. Diets were mixed in an orbital mixer (Kitchen Aid), extruded with a Kitchen Aid extruder (KPEXTA) and were air-dried before storage. The detailed compositions of the diets are provided in the Table S1.

The survival of fish was monitored up to 30 dpf. Then, surviving fish were collected and imaged at 30 dpf with an Axio Zoom.V16 Stereoscope (Zeiss) with ZEN pro 2012 software (Zeiss). Using the acquired images, fish standard lengths (Parichy et al., 2009) were

measured with ImageJ. To acquire normalized body length of *cubn* and *dab2* mutants at 5 and 30 dpf (Figure 5D), the standard lengths of 5 dpf and 30 dpf *cubn* or *dab2* mutants were divided by the average standard length of the same age *cubn* or *dab2* heterozygous controls, respectively.

### Immunofluorescence and Histology

Zebrafish larval and adult gut transverse 200-micron sections were cut with a vibratome (VT 1000S; Leica) and stained as described previously (Bagnat et al., 2007). In brief, larvae or adult gut were fixed in 4% PFA for 2 hours at room temperature or overnight at 4°C, washed in PBS, embedded in 5% low-melt agarose in PBS, sectioned with a vibratome and stained with an appropriate antibody. For immunofluorescence staining of mouse tissues, organs were embedded in OCT after perfusion with 4% PFA, frozen sections were collected using a Leica cryotome, and sections were stained as previously described (Sumigra et al., 2018). Briefly, sections were washed in PBS-T containing 0.2% Triton X-100, then incubated in blocking buffer (3% BSA, 5% NGS, 5% NDS in PBS-T) for 15 min. Sections were incubated in primary antibody diluted in blocking buffer for 15 min – 1 hr at room temperature. After washing in PBS-T, sections were incubated in secondary antibodies for 10 min, washed, then mounted for imaging. The following antibodies were used for immunohistochemistry: anti-Rab5 (GeneTex), anti-Rab7 (Cell Signaling), anti-Disabled-2/p96 (BD Bioscience), anti-LAMP2 (DSHB), anti-ATPase, (Na (+) K (+) alpha subunit (DSHB), anti- $\beta$ -catenin (Sigma). For histological analysis, mice were fixed by perfusion with 4 % PFA and then organs were harvested and fixed by immersion overnight. Tissues were then dehydrated in ethanol, infiltrated overnight, embedded in plastic resin the JB4-Plus kit (Polysciences), and 1-2 micron sections were cut using a Reichert-Jung UltraCut microtome equipped with a glass knife. Plastic sections were stained with Mayer's hematoxylin, eosin-Y, periodic acid-Schiff, or Oil Red O (Sigma), mounted with Cytoseal XYL, and imaged using either an Axioimager Z1 (Zeiss) or Axio Zoom.V16 Stereoscope (Zeiss).

### Ex Vivo MCherry Uptake in Mouse Intestinal Tissue

For uptake experiments, villin-CreER (el Marjou et al., 2004) *Dab2* cKO were used. The mom was injected with 5 mg tamoxifen in corn oil (Sigma) on P0 and P3, and pups were sacrificed at P6. Loss of protein was confirmed by immunofluorescence staining. Proximal and distal small intestines were dissected from P6 neonatal mice and washed with HBSS. 150  $\mu$ g of 20 mg/ml mCherry was injected into the lumen of a 2 cm segment of dissected intestine, and the intestine was cultured on a Transwell dish (Corning) containing L-WRN conditioned media for 1.5 hours at 37°C, flushed with HBSS and fixed in 4% PFA overnight. The intestines were then washed in PBS and embed in optimal cutting temperature (TissueTek), and 8- $\mu$ m sections were cut on a cryostat (Leica). Sections were allowed to dry, then fixed in 4% PFA for 8 min. Sections were then blocked in 5% normal goat serum, 5% Normal Donkey Serum and 3% bovine serum albumin in PBS + 0.2% Triton-X 100. Primary antibodies were incubated for 1 h at RT, followed by secondary antibodies for 10 min at RT. Slides were washed in PBS-T, then mounted in 90% glycerol in PBS plus 2.5 mg/ml *p*-Phenylenediamine (Sigma). Tissue sections were imaged on an Axioimager Z1 Microscope (Zeiss) with Apotome.2 attachment and 63X Plan-Apo 1.4 NA oil lens. Zen software (Zeiss) was used.

### QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism version 7.04. for Windows (GraphPad Software) and Microsoft Excel were used to analyze and plot data. Statistical analysis for the comparisons of larval survival rate (Figures 5A and 5B), larval body length measurement (Figures 5C and 6D) and mCherry accumulation in the pronephros (Figures S3C and S3D) was calculated from two-tailed unpaired t-tests in GraphPad Prism. Statistical analysis for *Dab2* WT and cKO mouse data including body length (Figure 7K), body weight (Figure 7L), average weight gain/day (Figure 7M) and duodenum diameter (Figure 7O) was calculated from two-tailed paired t-tests in GraphPad Prism. Information and measurements of individual *Dab2* WT and cKO mice are provided in the Table S2. Exact p-values, t-values and degree of freedom for all the t-tests are provided in the Table S3. Sample size for each experiment is indicated in the figure legend for each experiment.

### DATA AND CODE AVAILABILITY

Raw and processed data for the RNA-seq experiment (Figures 2A, 2B, and S4) are available in GEO DataSets with the following accession number: GSE124970.