HHS Public Access

Author manuscript

Dev Biol. Author manuscript; available in PMC 2015 April 29.

Published in final edited form as:

Dev Biol. 2011 August 15; 356(2): 588–597. doi:10.1016/j.ydbio.2011.06.024.

UVRAG is required for organ rotation by regulating Notch endocytosis in *Drosophila*

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Abstract

Heterotaxy characterized by abnormal left–right body asymmetry causes diverse congenital anomalies. Organ rotation is a crucial developmental process to establish the left–right patterning during animal development. However, the molecular basis of how organ rotation is regulated is poorly understood. Here we report that *Drosophila UV-resistance associated gene* (*UVRAG*), a tumor suppressor that regulates autophagy and endocytosis, plays unexpected roles in controlling organ rotation. Loss-of-function mutants of *UVRAG* show seriously impaired organ rotation phenotypes, which are associated with defects in endocytic trafficking rather than autophagy. Blunted endocytic degradation by UVRAG deficiency causes endosomal accumulation of Notch, resulting in abnormally enhanced Notch activity. Knockdown of Notch itself or expression of a dominant negative form of Notch transcriptional co-activator Mastermind is sufficient to rescue the rotation defect in *UVRAG* mutants. Consistently, *UVRAG*-mutated heterotaxy patient cells also display highly increased Notch protein levels. These results suggest evolutionarily conserved roles of UVRAG in organ rotation by regulating Notch endocytic degradation.

Keywords

UVRAG; Organ rotation; Vesicle trafficking; Notch endocytosis; Left – right body asymmetry

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Appendix A. Supplementary data: Supplementary data to this article can be found online at doi:10. 1016/j.ydbio.2011.06.024.

Introduction

Internal organs such as heart, liver and gut undergo a directional rotation process to establish left–right body asymmetry during animal development (Palmer, 2004). For example, in humans, the heart undergoes dextral rotation to be ultimately located in the left side of the body. Heterotaxy, which involves non-rotation, reverse rotation and mal-rotation of internal organs, leads to various syndromes and pathologies including asplenia, polysplenia, congenital heart defects and early fetal death, indicating importance of the rotation process in organ development and function (Belmont et al., 2004; Bisgrove et al., 2003).

The organ rotation around a longitudinal body axis is an evolutionarily conserved process from worms to humans (Speder et al., 2007). In *Drosophila melanogaster*, the looping of embryonic gut and 360° dextral rotation of adult male genitalia with spermiduct looping are the two representative organ rotation programs (Coutelis et al., 2008; Okumura et al., 2008). Previous studies in *Drosophila* have discovered several genes involved in organ rotation such as *Fasciclin II* (Adam et al., 2003), *JNK* (Macias et al., 2004; McEwen and Peifer, 2005; Taniguchi et al., 2007), *Myosin ID* (Hozumi et al., 2006; Speder et al., 2006) and *single-minded* (Maeda et al., 2007). For instance, in the mutants of *Fasciclin II* that regulates juvenile hormone metabolism in central nervous system, the genitalia rotation is incomplete while the direction of rotation is normal (Adam et al., 2003). On the other hand, mutations of the actin-based motor protein *Myosin ID* lead to complete reversion of the looping direction (Hozumi et al., 2006; Speder et al., 2006). The molecular mechanisms of how all these seemingly divergent genes orchestrate organ rotation remain to be elucidated.

UVRAG was initially identified for its complementary effect on UV sensitivity in xeroderma pigmentosum cells (Perelman et al., 1997). Genetic association studies have shown that the human chromosomal region containing *UVRAG* is closely associated with the pathogenesis of various human cancers and heterotaxy syndromes (Bekri et al., 1997; Goi et al., 2003; Iida et al., 2000; Ionov et al., 2004; Kosaki and Casey, 1998). Recent biochemical and cell biological studies in mammalian cells have demonstrated that UVRAG interacts with Atg6 and class C vacuolar protein sorting complexes, thereby regulating both autophagy and vesicle trafficking (Itakura et al., 2008; Liang et al., 2006, 2008). Despite these advances inour understanding of UVRAG functions at the molecular level, physiological and developmental roles of UVRAG have not been investigated yet.

Vesicle trafficking controls a variety of intracellular processes including protein turnover and protein targeting to different organelles. In particular, endocytic trafficking pathway modulates localization of membrane signaling proteins to specific intracellular vesicle compartments as well as their lysosomal degradation to achieve the fine tuning of extracellular signals and cell homeostasis (Deretic, 2005; Gonzalez-Gaitan, 2003; Seto et al., 2002; Sorkin and von Zastrow, 2009). In fact, several loss-of-function mutants of endocytic trafficking genes have been shown to exhibit dysregulated cell survival and proliferation (Gonzalez-Gaitan and Stenmark, 2003; Herz and Bergmann, 2009; Vaccari and Bilder, 2009). Recently, endocytic trafficking has also emerged as a crucial regulatory mechanism for animal body development. Expression levels of numerous endocytic trafficking genes are dynamically altered during *Drosophila* metamorphosis (Lee et al., 2003; Li and White,

2003; Martin et al., 2007), and mutations of endocytic trafficking genes cause severe developmental defects in mammals (Cheng et al., 2006; Dell'Angelica, 2009; Sato et al., 2007). However, it is still unknown whether endocytic trafficking plays important roles in organ rotation.

In this study, we have generated *Drosophila UVRAG* loss-of-function mutants and identified unexpected roles of UVRAG in regulating organ rotation. We found that UVRAG is important for organ rotation by regulating receptor endocytosis and subsequent degradation rather than autophagy induction. Moreover, our results show that Notch is the key downstream target regulated by UVRAG in both *Drosophila* and human cells, implicating an evolutionarily conserved role of UVRAG in Notch signaling regulation and organ rotation.

Results

Identification of UVRAG as a novel cell growth regulator

We performed a *Drosophila* genetic screen using P-element lines that show homozygous lethality to identify novel cell growth regulators. By generating mosaic clones (Xu and Rubin, 1993) of P-element lines in adult ovaries, we identified *GS17330* allele which showed highly increased number of follicle cells. In contrast to the typical cuboidal and monolayered wild type follicle cells (Fig. 1A, left), GFP-negative *GS17330* mosaic clones were mostly round-shaped and multilayered (Fig. 1A, right), suggesting that the *GS17330* allele affects a potential cell growth regulator gene (Bilder et al., 2000; Goode and Perrimon, 1997; Tepass et al., 2001).

The P-element of *GS17330* was inserted in the 5′ untranslated region (UTR) of a previously uncharacterized gene, *CG6116* (FlyBase ID; FBgn0032499) (Fig. 1B). BLAST search analyses indicated that *CG6116* is a *Drosophila* ortholog of *UVRAG* (Supplemental Fig. S1). Using imprecise excision of the P-element of another *UVRAG* mutant *KG04163*, we generated two deletion mutants of *UVRAG*, $UVRAG^{B7}$ and $UVRAG^{B21}$ (Fig. 1B), in which *UVRAG* transcripts were not detected by RT-PCR (Fig. 1C). The deletion mutants and their trans-heterozygotes combined with a deficiency line covering *UVRAG* were all larval lethal (Fig. 1D), but the lethality was rescued by transgenic expression of *UVRAG* under ubiquitous *daughterless* (*da*)-*Gal4* driver (Fig. 1D). Similar to the *GS17330* clones (Fig. 1A), *UVRAG* null mutant clones also showed active cell proliferation (Supplemental Fig. S2). These results demonstrate that *Drosophila* UVRAG is required for normal fly development and cell growth regulation.

UVRAG is required for organ rotation

Since *UVRAG* null mutant is early larval lethal, we employed an adult-viable *UVRAG* hypomorphic allele *KG04163* (Figs. 1B–D) to investigate the roles of UVRAG in later development. Strikingly, compared to wild type, ∼50% of *KG04163* males showed abnormal genitalia orientation (Fig. 2A and Table 1). In wild type flies, genitalia undergoes a complete 360° dextral rotation and induces looping of the spermiduct around the gut (Figs. 2A and B, left panels), which is comparable to the directional looping of internal organs in

vertebrates (Speder et al., 2007). The spermiduct of *KG04163* did not coil around the gut (Fig. 2B, right), suggesting that the failure in genitalia rotation leadsto impaired gut looping. The incomplete rotation phenotype was severed by lowering *UVRAG* gene dosage, as shown by a much lower rotation degree of *KG04163* trans-heterozygotes with *UVRAG* null alleles ($UVRAG^{KG/B7}$ and $UVRAG^{KG/B21}$) compared to that of *KG04163* homozygotes (Fig. 2C compared to Fig. 2A and Table 1). However, the direction of rotation in *UVRAG* mutants was constantly dextral (Fig. 2 and data not shown), indicating that UVRAG is not involved in the determination of organ rotation direction.

Interestingly, transgenic expression of *UVRAG* (Supplemental Fig. S3A) using genitalia-specific *Abdominal B* (*AbdB*)-*Gal4* (de Navas et al., 2006; Speder et al., 2006) was sufficient to rescue the rotation defect in *UVRAG* mutants while central nervous system (*elav-Gal4*)- or fat body (*Lsp2-Gal4)*-specific expression did not (Fig. 2D, left and Table 1). These results showed a tissue-specific role of UVRAG in regulating organ rotation.

Quantitative RT-PCR analyses revealed that *UVRAG* expression is highest at the pupa stage (Supplemental Fig. S3B), and transient expression of *UVRAG* from late larval to early pupa stage (7±2 days after egg laying, AEL) sufficiently rescued the rotation defect in *UVRAG* mutants (Fig. 2D, right). This developmental stage-selective UVRAG function in organ rotation is consistent with the previously described *Drosophila* looping morphogenesis (Adam et al., 2003; Speder et al., 2006). Collectively, these results indicate that UVRAG plays crucial roles in organ rotation process during *Drosophila* development.

Autophagy may not be involved in the organ rotation process

Since mammalian UVRAG is known to regulate autophagy (Itakura et al., 2008; Liang et al., 2006, 2008; Zhong et al., 2009), we examined whether the organ rotation defect in *UVRAG* mutants is caused by impaired autophagy. We observed that the lysotracker staining and localization of Atg8, autophagy markers which localize to autophagosomes, showed punctuate patterns in wild type cells but not in *UVRAG* null cells in starved larval fat body in which autophagy occurs actively (Supplemental Fig. S4A) (Chang and Neufeld, 2009; Levine and Klionsky, 2004; Rusten et al., 2007). However, both wild type and *UVRAG* mutant larval genital discs showed dispersed Atg8 localization in the cytoplasm (Supplemental Fig. S4B), suggesting that autophagy does not actively occur in genital discs. Moreover, the cleavage of Atg8, which occurs during autophagy process (Klionsky et al., 2008; Rusten et al., 2004), was observed in wandering larva fat body while that was not observed in the pupa genitalia of both wild type and *UVRAG* mutant flies (Supplemental Fig. S4B).

We then examined whether the mutations of critical autophagy regulators cause rotation defects. Interestingly, loss-of-function mutants of *Atg1* (Lee et al., 2007; Scott et al., 2004), *Atg5* (Scott et al., 2004), *Atg6* (Scott et al., 2004) and *Atg7* (Juhasz et al., 2007) exhibited normal organ looping and genitalia orientation in contrast to *UVRAG* mutants (Supplemental Fig. S4C and Table S1). Collectively, these results suggest that autophagy does not account for the organ rotation defect in *UVRAG* mutants.

UVRAG mutant cells show defective endocytic degradation of signaling proteins

We next examined whether the organ rotation defects in *UVRAG* mutants is related to endocytic trafficking since UVRAG is also known to function in endocytosis (Itakura et al., 2008; Liang et al., 2008). Surprisingly, loss-of-function mutants of the genes for endocytic trafficking such as *Rab5* (Lu and Bilder, 2005) and *vps25* (Herz et al., 2006, 2009; Thompson et al., 2005; Vaccari and Bilder, 2005) showed incomplete genitalia rotation phenotypes similar to *UVRAG* mutants (Fig. 3A, Table 1 Supplemental Fig. S5A), strongly suggesting that the rotation defect in *UVRAG* mutants is due to impaired endocytosis.

To assess this possibility, we examined the cellular localization of several membrane proteins known to be regulated by endocytic trafficking. Epidermal growth factor receptor (EGFR), Notch, Patched (Ptc) and PDGF/VEGF receptors (PVR) showed much stronger signals in GFP-negative *UVRAG* null clones than those in surrounding GFP-positive control cells (Fig. 3B, upper and middle). However, this was not the case for Fasciclin III (FasIII) and E-Cadherin (E-Cad) (Shilo, 1992; Tepass et al., 2001) (Fig. 3B, lower), implying that UVRAG primarily functions in stimulating endocytic trafficking of signaling receptors rather than cell adhesion proteins.

The enhanced signals of the receptors in *UVRAG* null clones showed irregular punctate structures (inlets in the upper and middle panels of Fig. 3B). By co-staining with organelle markers, we observed that the accumulated Notch was barely co-localized to the actin-enriched plasma membrane (Fig. 3C, upper) but markedly co-localized with the endosome marker Hrs (Jekely and Rorth, 2003; Lloyd et al., 2002) (Fig. 3C, lower). These data indicate that Notch is abnormally accumulated in endosomes in the absence of UVRAG. To further investigate the mechanism of receptor accumulation in *UVRAG* null cells, we performed time-course experiments in live larval discs using an antibody against the extracellular domain of Notch (Le Borgne and Schweisguth, 2003). In GFP-positive control cells, the cell surface-localized Notch proteins were internalized and disappeared within 5 h after chasing (Fig. 3D). On the other hand, Notch proteins in *UVRAG* null clones were internalized normally but trapped in vesicular structures even at 5 h of chasing (Fig. 3D). Consistently, ubiquitin known to be conjugated to the membrane receptors for lysosomal targeting and degradation (Jekely and Rorth, 2003; Katzmann et al., 2002) was also highly accumulated in *UVRAG* null clones (Fig. 3E). Collectively, these data show that UVRAG is required for the endocytic degradation of membrane-localized receptor proteins.

Notch is the key downstream target of UVRAG

We next examined genetic interactions between UVRAG and the receptors accumulated in *UVRAG* null cells (Fig. 3). While wing-specific *UVRAG* knockdown induced ruffling of wings (Supplemental Figs. 6A and B) (Hipfner and Cohen, 2003; Morrison et al., 2008), downregulation of *Notch* (Presente et al., 2002) alone was sufficient to suppress this phenotype (Supplemental Figs. 6A and B). However, downreglation of *EGFR*, *Ptc* or *PVR* (Supplemental Fig. S5B) (Rosin et al., 2004) was not (Supplemental Fig. 6A). Furthermore, the semi-lethality and increased number of wing hair cells in UVRAG-deficient flies were considerably relieved by *Notch* downregulation but enhanced by *Notch* over expression (Supplemental Figs. 6 and 7). Consistent with these genetic interaction data, *UVRAG*

knockdown strongly enhanced the expression of Notch reporter, *Notch Response Element* (*NRE*)-*EGFP* (Saj et al., 2010) (Supplemental Fig. S6C), implying that Notch signaling is highly activated in UVRAG-deficient cells. Furthermore, the follicle cell proliferation and degenerated eyes of *UVRAG* null clones were also significantly rescued by *Notch* knock down (Supplemental Fig.S8). These specific and strong genetic interactions between UVRAG and Notch suggest that Notch is the key downstream target of UVRAG.

UVRAG regulates organ rotation by inhibiting Notch activity

We then assessed whether the organ rotation defect in *UVRAG* mutants is also caused by deregulated Notch signaling. We observed that downregulation of *Notch* rescued the rotation defect in *UVRAG* mutants, whereas downregulation of *EGFR*, *Ptc* or *PVR* did not (Table 1 and Fig. 4). In addition, the genital discs of *UVRAG* mutant showed much stronger punctate Notch signals and increased Notch protein level than that of wild type (Figs. 4A and B) (Acar et al., 2008; Vaccari et al., 2008). Transgenic *UVRAG* expression suppressed Notch accumulation (Fig. 4A) and rescued the rotation defect in *UVRAG* mutants (Fig. 2D and Table 1). The genital discs in *UVRAG* mutant exhibited increased expression of Notch reporter (*NRE-EGFP*) (Fig. 4C), indicating enhanced Notch activity in *UVRAG* mutant's genitalia.

Furthermore, inhibition of Notch activity by expression of a dominant negative form of Mastermind, the Notch transcription co-activator (Kankel et al., 2007; Wu et al., 2000), rescued the genitalia rotation defect in *UVRAG* mutant (Fig. 4D and Table 1). Collectively, these data indicate that *UVRAG* regulates organ rotation by inhibiting Notch signaling.

To examine whether the UVRAG's function in Notch regulation and organ rotation is also conserved in humans, we compared the amount of endogenous Notch1 proteins in human cells isolated from a normal patient and a heterotaxy patient with a monoallelic disruption of *UVRAG* (Iida et al., 2000). As observed in *Drosophila* (Figs. 3 and 4), UVRAG-deficient heterotaxy patient's cells showed a significantly increased level of Notch1 (Fig. 5A). Furthermore, the amount of Notch1 was highly detected in human colon cancer HCT116 cells with a monoallelic loss of *UVRAG* (Liang et al., 2006) (Fig. 5B), but it was reduced upon the complementation of *UVRAG* expression (Fig. 5B). These data strongly suggest an evolutionarily conserved role of UVRAG to keep Notch signaling at appropriate levels in human cells as in *Drosophila* (Fig. 6).

Discussion

Genetic analyses in human patients have indicated that *UVRAG* is encoded in one of the 17 chromosomal loci linked with heterotaxy (Iida et al., 2000; Kosaki and Casey, 1998), a condition showing defective pattern of typical left–right asymmetry for internal organs. In the present study, we found organ rotation defects in *Drosophila UVRAG* mutants, suggesting the evolutionarily conserved function of UVRAG in left–right body asymmetry formation from flies to humans.

Mammalian UVRAG has been known for regulating autophagy and vesicle trafficking (Itakura et al., 2008; Liang et al., 2006, 2008). Interestingly, our data showed that UVRAG's

role in organ rotation was regulated specifically by endocytic trafficking rather than autophagy. Loss-of-function mutants of critical autophagy-regulating genes such as *Atg1*, *Atg5*, *Atg6* and *Atg7* showed normal organ rotation (Supplemental Fig. S4). In contrast, disruption of endocytic trafficking process by a loss-of-function mutation of *Rab5* or *vps25* caused incomplete organ rotation similar to *UVRAG* mutants (Fig. 3). As the mutations in these different components of vesicle trafficking pathway result in similar organ rotation failures in *Drosophila*, we strongly believe that some specific molecules delivered by cytosolic vesicles play crucial roles in the formation of left–right body asymmetry. Indeed, Myosin ID, the molecular motor protein, has been also suggested to control the direction of organ rotation by delivering specific intracellular cargos or vesicles (Hozumi et al., 2006; Speder et al., 2006).

The specific genetic interaction of UVRAG with Notch among several signaling proteins indicates that Notch is the key physiological target regulated by UVRAG. As shown by the receptor chasing assay in live imaginal discs, Notch was sufficiently removed from the plasma membrane to intracellular vesicles but constantly trapped in endosomes in *UVRAG* null cells (Fig. 3). Consistently, ubiquitin that labels membrane proteins destined for lysosomal degradation was also highly accumulated in *UVRAG* null cells (Fig. 3). These data suggest that *UVRAG* is required for the late endocytic trafficking or subsequent targeting of Notch to lysosomes.

Intriguingly, Notch signaling is increased in UVRAG-deficient cells (Fig. 4 and Supplemental Fig. S6). One possibility is that the endosome-accumulated Notch is activated via the increased accessibility of γ-secretase, which cleaves and releases an active form of Notch (Fortini, 2002; Pasternak et al., 2004). Nullifying the interaction of Notch with its extracellular ligands by expression of an extracellular domain of Notch ($Notch^{ECN}$) (Acar et al., 2008) did not rescue the rotation defect in *UVRAG* mutants (Table 1). On the other hand, inhibiting Notch activity by expressing a dominant negative form of Notch transcription co-activator, Mastermind (Kankel et al., 2007; Wu et al., 2000), did rescue the *UVRAG* mutant phenotype (Fig. 4 and Table 1). This is also in agreement with the previous studies suggesting that the endosomal activation of Notch occurs in a ligand-independent manner (Baron, 2003; Fortini, 2009; Vaccari et al., 2008).

The organ rotation defect in *UVRAG* mutants was rescued by *Notch* knockdown (Fig. 4 and Table 1). Conversely, expression of transgenic *Notch* impaired the genitalia rotation similar to *UVRAG* mutants (Table 1), and expression of a constitutively active form of *Notch* ($Notch^{ICD}$) (Acar et al., 2008) caused a much more severe phenotype (Table 1). In line with these data, mutations of Notch signaling in *C. elegans*, zebrafish, chicken and mouse have also been reported to cause defects in left–right patterning (Hermann et al., 2000; Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2003, 2004). Thus, the precise regulation of Notch signaling might be an evolutionarily conserved factor for the left–right body asymmetry formation (Lai, 2004).

Then how does Notch control body asymmetry formation? Since *UVRAG* regulates tissue growth via *Notch* signaling (Supplemental Figs. S6–S8), it is possible that the tissue enlargement nonspecifically caused organ rotation defect. However, we observed that

expression of other cell growth regulators caused no or mild effect on the rotation even though they showed the increased tissue size similar to or even more seriously than *UVRAG* mutant (Supplemental Table S1 and Fig. S9). Meanwhile, *Notch* directly controls the expression of left–right patterning genes in other species; mouse (*nodal*; TGF-beta-like protein) (Krebs et al., 2003; Raya et al., 2003), zebrafish (*charon*; BMP antagonist protein) (Lopes et al., 2010) and *Xenopus* (*pitx2*; homeobox protein) (Sakano et al., 2010). In the similar manner, we examined the transcriptional profiles of several genes related to organ rotation in *Drosophila* such as JNK signaling molecules (*puckered* and *scarface*) (Macias et al., 2004; Rousset et al., 2010), apoptosis-related genes (*dronc*, *hid* and *DIAP*) (Abbott and Lengyel, 1991; Krieser et al., 2007; Suzanne et al., 2010) and actin cytoskeleton regulating genes (*drac1* and *cdc42*) (Speder et al., 2006). Unfortunately, we could not observe significant changes in their expression levels both in *UVRAG* and *Notch* mutants (Supplemental Fig. S10). However, we still believe that there must be direct Notch downstream target genes regulating left–right body patterning in *Drosophila*.

In conclusion, the first knockout animexpression levels both in al model of *UVRAG* in this study recapitulates important aspects of *UVRAG*-mutated heterotaxy syndrome such as failure in the rotation of left–right asymmetric organs and severe developmental defects. Mechanistically, we also firstly demonstrated that UVRAG negatively regulates Notch activity by endocytic degradation in both flies and humans. Our findings suggest that Notch signaling can be a potential therapeutic target for treating *UVRAG*-mutated heterotaxy syndrome.

Material and methods

Drosophila genetics

$UVRAG^{B7}$ and $UVRAG^{B21}$ were generated by imprecise excisions of the P-element in *KG04163* allele (Bloomington Stock Center). Genomic lesions were determined by genomic PCR and sequencing using primers: 5′-GCAGCTGTTGCCATTCTCCGAATAGG-3′ and 5′-GTTATGCTC-CAGTCGCGGGCG-3′. The following stocks were kindly provided by other groups: *AbdB-Gal4* (a gift from Dr. Ernesto Sanchez-Herrero, Universidad Autonoma de Madrid, Spain), *UAS-Notch* and $UAS\text{-}Notch^{ECN}$ (gifts of Dr. Hugo Bellen, Baylor College of Medicine, USA; originally generated by Dr. Gary Struhl, Columbia University College of Physicians and Surgeons, USA), *hsFLP*; $ck^{13}FRT40A$ (a gift from Dr. Kyung-Ok Cho, Korea Advanced Institute of Science and Technology; originally generated by Dr. Hugo Bellen, Baylor College of Medicine, USA), *hh-Gal4* (a gift from Dr. Masayuki Miura, University of Tokyo, Japan; originally generated by Dr. Tetsuya Tabata, University of Tokyo, Japan), *UAS-GFPAtg8* (a gift from Dr. Harald Stenmark, University of Oslo, Norway), $Atg7^{d14}$, $Atg7^{d77}$, $UAS\text{-}Atg5^{RNAi}$, *UAS-mCherryAtg8* and *hsFLP*; *UAS-2XeGFP FRT40A fb-Gal4* (gifts of Dr. Thomas Neufeld, University of Minnesota, USA), $UAS\text{-}Pvr^{RNAi}$ (a gift of Dr. Ben-Zion Shilo, Weizmann Institute of Science, Israel), $UAS\text{-}Notch^{ICD}$ (a gift of Dr. Jaeseob Kim, Korea Advanced Institute of Science and Technology), $UAS\text{-}UVRAG^{RNAi}$ and $UAS\text{-}Ptc^{RNAi}$ (National Institute of Genetics, Japan), *GS17330* (*Drosophila* Genetic Resource Center, Kyoto Institute of Technology, Japan), $UAS\text{-}Rab5^{RNAi}$, $UAS\text{-}vps25^{RNAi}$ and $UAS\text{-}EGFR^{RNAi}$ (VDRC Stock Center, Vienna *Drosophila*

Research Center, Austria). Other stocks were obtained from Bloomington Stock Center (Indiana University, USA) or described elsewhere (Kim et al., 2006; Lee et al., 2007; Lee and Chung, 2007).

GS17330 and *UVRAG*^{B21} were recombined with *FRT40A* to generate mosaic clones. For generating clones in adult ovary, flies were heat-shocked in 37 °C water bath for 1 h to stimulate the heat-shock inducible flippase at the second day after eclosion and dissected on the fifth day after eclosion. To generate clones in fat body, eggs were collected for 8 h and followed by heat-shock for 2 h. Wing mosaic clones were generated by 1 h heat-shock at the second day after egg laying. Mosaic eye clones were generated by flippase expressed by *eyeless* gene promoter (Tapon et al., 2001). For rescue experiments using *hs-Gal4*, eggs were collected for 4 h everyday for subsequent 11 day and heat-shocked for 1 h at the 11th day (Adam et al., 2003). For amino acid starvation, early third instar larvae were incubated for 4 h in wet kimwipes containing 20% (w/v) sucrose in PBS. Flies were raised at 25 °C on standard cornmeal/sucrose/yeast/agar media unless otherwise indicated.

Cell culture

Epstein–Barr virus (EBV)-immortalized human B cells (gifts from Dr. Hirofumi Ohashi, Saitama Children's Medical Center, Japan) and HCT116 and HEK293T cells were cultured in RPMI-1640 (Sigma-Aldrich) and Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), respectively, supplemented with 10% (v/v) FBS (Invitrogen), 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco-BRL) at 37 °C under 5% CO_2. HCT116 stable cell lines expressing an empty vector and Flag-human UVRAG are previously described (Liang et al., 2006).

Antibodies

The following antibodies were used: Anti-Hrs (a gift from Dr. Hugo Bellen, Baylor College of Medicine, USA), anti-EGFR (a gift from Dr. Pernille Rorth, Temasek Life Sciences Laboratory, Singapore), anti-PVR (a gift from Dr. Denise Montell, Johns Hopkins School of Medicine, USA), anti-mono and poly ubiquitinylated proteins (clone No. FK2 and PW8810; Biomol), anti-beta-Tubulin, anti-Notch intracellular domain, anti-Notch extracellular domain, anti-Patched, anti-Fasciclin III, anti-E-Cadherin (E7, C17.9C6, C458.2H, APA1, 7G10, DCAD2; Developmental Studies Hybridoma Bank, The University of Iowa, USA), anti-Flag, anti-human UVRAG, anti-Actin (F1804, U7508, A-3853; Sigma-Aldrich), anti-Notch1 (C-20), anti-GFP (sc-6014, sc-8334; Santa Cruz Biotechnology) antibodies. Anti-*Drosophila* UVRAG antibody was generated by immunizing rabbits with the peptide, CRYIERTQRDEVDERDGT-NH2 (Peptron, Korea).

Histology

Immunostaining analyses for tissues and cells were performed as previously described (Kim et al., 2006; Liang et al., 2008). TRITC-labeled phalloidin (Sigma-Aldrich) and Hoechst 33258 (Sigma-Aldrich) were used to visualize filamentous actin and DNA, respectively. For endocytic trafficking assays, larval eye imaginal discs were incubated with anti-Notch extracellular domain antibody in M3 medium at 4 °C for 5 min and chased for 5 h at 25 °C as previously described (Le Borgne and Schweisguth, 2003; Vaccari et al., 2008).

LysoTracker staining was conducted as previously described (Juhasz et al., 2008; Scott et al., 2004). Briefly, fat body was fixed for 3 min in 2% paraformaldehyde in PBS, rinsed with PBS, incubated with 100 nM LysoTracker Red DND-99 (Invitrogen) for 2 min, and then mounted using PBS. For mCherry-Atg8 or GFP-Atg8 detection, fat body or imaginal discs were mounted using 3% DABCO (Sigma-Aldrich) in 90% (v/v) glycerol in PBS. Adult wings were mounted in Canada balsam (Sigma-Aldrich):Methyl salicylate (Sigma Aldrich) (2:1).

Statistical analyses were performed using the Student's *t* test. Values are expressed as mean s.e.m. of at least three independent experiments.

Molecular biology

To generate transgenic flies, the full-length *UVRAG* (*CG6116*) cDNA (*LD05963; Drosophila* Genomics Resource Center) was cloned into Flag-tagged pUAST vector, and microinjected into w^{1118} embryos as previously described (Kim et al., 2006).

Immunoblot analyses for tissues and cells were performed as previously described (Kim et al., 2006; Liang et al., 2008). For immunoblot analyses of Notch in flies, samples were incubated for 20 min on ice in the buffer containing 10 mM KCl, 20 mM Tris (pH 7.5), 0.1% mercaptoethanol,1 mM EDTA with complete protease inhibitor cocktail. For immunoblot analyses of GFP-LC3 in flies, samples were lysed directly in SDS sample buffer.

RT-PCR and quantitative RT-PCR analyses were performed as previously described (Lee et al., 2007). The following primers were used for PCR:

	Forward	**Reverse**
rp49	5′-GCTTCAAGATGACCATCCGCCC-3′	5′-GGTGCGCTTGTTCGATCCGTAAC-3′
Actin	5′-CGTCTTCCCATCGATTGTG-3′	5′-GATGCCAGGGTACATGGTG-3′
UVRAG (RT-PCR)	5′-CCAACGGCAGGAGATTCGGCGAAGC-3′	5′-GAGGTCGATCCATAATGATGGCTAAC-3′
UVRAG (qRT-PCR)	5′-CGTCTGGAGCTACGAACCCTGG-3′	5′-GCTCCATGTGGGGGAAGGCG-3′
Rab5	5′-GAAGCAATATGCCGAGGAGA-3′	5′-CAAATGAAATTCGTCCCCTG-3′
vps25	5′-CACCTTCCCACCCTTCTTT-3′	5′-CATCTCGATCAGCTCACAC-3′
EGFR	5′-GAGCTGGAGCAGATCACT-3′	5′-AGTGCAACCGTTGCATTC-3′
Ptc	5′-ATCGTAATGTGCTCCAATTTG-3′	5′-GAGCCGAGTTTGAGCATC-3′
dronc	5′-CTGGCTTTGGTGCCGTCAATTATCC-3′	5′-TTGCGCTGGACCGCAGAAGC-3′
hid	5′-ACCACCTCGTCGGCCACGCAGA-3′	5′-GGGTGCGCGGATGGGGATTC-3′
diap	5′-GATGGTCGCCCAACTGTCCACTG-3′	5′-ACACTGCCTGCCGCATTTACTGC-3′
puckered	5′-ACAACAACAATCGCATTGGTGCCAATC-3′	5′-CCATTGCCCAGCAATAGATGCGG-3′
scarface	5′-ACGGCGAAATTAGCGCCATAAACTACG-3′	5′-GAAGCTGGCACAGCAGTCGTAGG-3′
drac1	5′-GCCACTGTCTTATCCCCAGACCG-3′	5′-GTGATGGGCGCCAGTTTCTTGTC-3′
cdc42	5′-CGGTGGTCAGTCCCAGTTCCTT-3′	5′-CACTCCACGTACTTGACGGCCTT-3′

Microscopy

Confocal images were acquired using a LSM 510 or 710 confocal microscopes with LSM image browser v.3.2 SP2 software (Carl Zeiss). Other microscopy images were acquired using a digital camera (AxioCam) with AxioVS40AC v.4.4 software (Carl Zeiss) and a light microscopy (Leica). Scanning electron microscopy images were obtained by LEO1455VP (Carl Zeiss) or SUPRA55VP (Carl Zeiss) in a variable pressure secondary electron mode. Images were processed in Photoshop v.7.0 (Adobe).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Ben-Zion Shilo, Denise Montell, Ernesto Sanchez-Herrero, Gary Struhl, Harald Stenmark, Hirofumi Ohashi, Hugo Bellen, Jaeseob Kim, Masayuki Miura, Pernille Rorth, Tetsuya Tabata and Thomas Neufeld for reagents and *Drosophila* lines. We are grateful to Drs. Kyung-Ok Cho, Kwang-Wook Choi and Young-YunKong for helpful discussion and kind support. We thank Bloomington Stock Center, Developmental Studies Hybridoma Bank, *Drosophila* Genetic Resource Center, *Drosophila* Genomics Resource Center, National Institute of Genetics and VDRC Stock Center for *Drosophila* stocks and antibodies, and Korea Basic Science Institute and National Instrumentation Center for Environmental Management (Seoul National University) for electron microscopic analysis. This work was supported by the National Creative Research Initiatives Program (2010-0018291) to J.C. and the Priority Research Centers Program (2009-0094022) to G.L. from the National Research Foundation (NRF) grant funded by the Ministry of Education, Science, and Technology (MEST) of Korea. J.J. was supported by U.S. Public Health Service grants CA82057, CA91819, CA31363, CA115284, AI073099, the Fletcher Jones Foundation, the Hastings Foundation and the Korean GRL Program (K20815000001). C.L. was supported by AI083841, CA140964, the Lymphoma and Leukemia Society of USA, the Wright Foundation and the Baxter Foundation.

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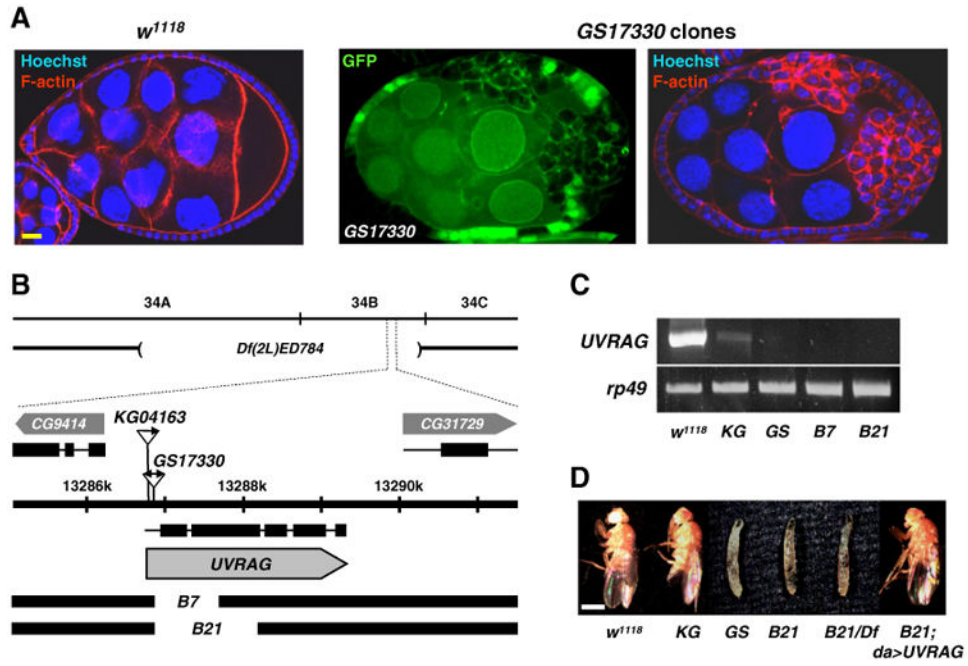

Fig. 1.

UVRAG is identified as a novel cell growth regulator. (A) Wild type (w^{1118}) ovary (left) and *GS17330* clone-containing ovary (right) were stained with TRITC-phalloidin (F-actin) and Hoechst 33258 (blue). Absence of GFP marks *GS17330* clones. (B) A schematic representation of the *UVRAG* genomic locus and deletion regions of $UVRAG^{B7}$(*B7*) and $UVRAG^{B21}$(*B21*). (C) RT-PCR analyses of *UVRAG* in wild type, *UVRAG* P-element insertion [$UVRAG^{KG}$ (*KG*) and $UVRAG^{GS}$ (*GS*)] and deletion (*B7* and *B21*) mutants. *rp49* was used as a loading control. (D) The lethality of *UVRAG* null mutants was rescued by transgenic expression of *UVRAG.Df,Df(2L)ED784*. Scale bars: yellow, 10 μm; white, 0.5 mm. Genotype: (A) Right: *hsFLP/+*; *GS17330 FRT40A/FRT40A UbiGFP*.

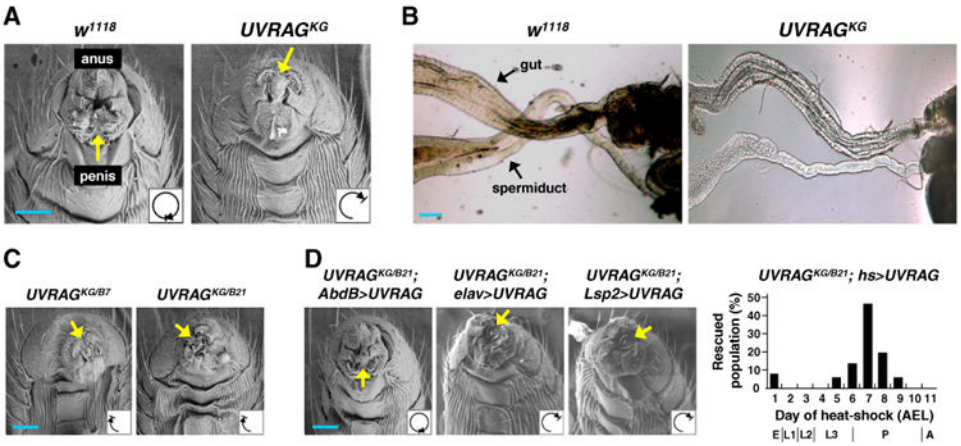

Fig. 2.

UVRAG is required for the adult organ rotation. (A, C, D) Ventral side views of adult male genitalia were visualized by scanning electron microscopy (SEM; posterior is upward). Yellow arrows indicate the location of penis. A schematic diagram for the direction and extent of genitalia rotation is indicated by the looping arrow (lower right inlets). The effect of tissue-specific or developmental stage-specific expression of transgenic *UVRAG* on the $UVRAG^{KG/B21}$ rotation phenotype was visualized by SEM (D, left three panels) or quantified throughout the development (D, graph in the right panel): embryo (E), first, second and third instar larva (L1, L2, L3), pupa (P) and adult (A). N >50 male flies for each genotype. AEL, after egg laying. (B) Dissected adult abdominal organs were visualized by light microscopy. Wild type shows the rightward looping of the spermiduct around the gut (left), while *UVRAG* mutant displays impaired looping (right). The strongest phenotype is shown. Scale bars, 50 μm.

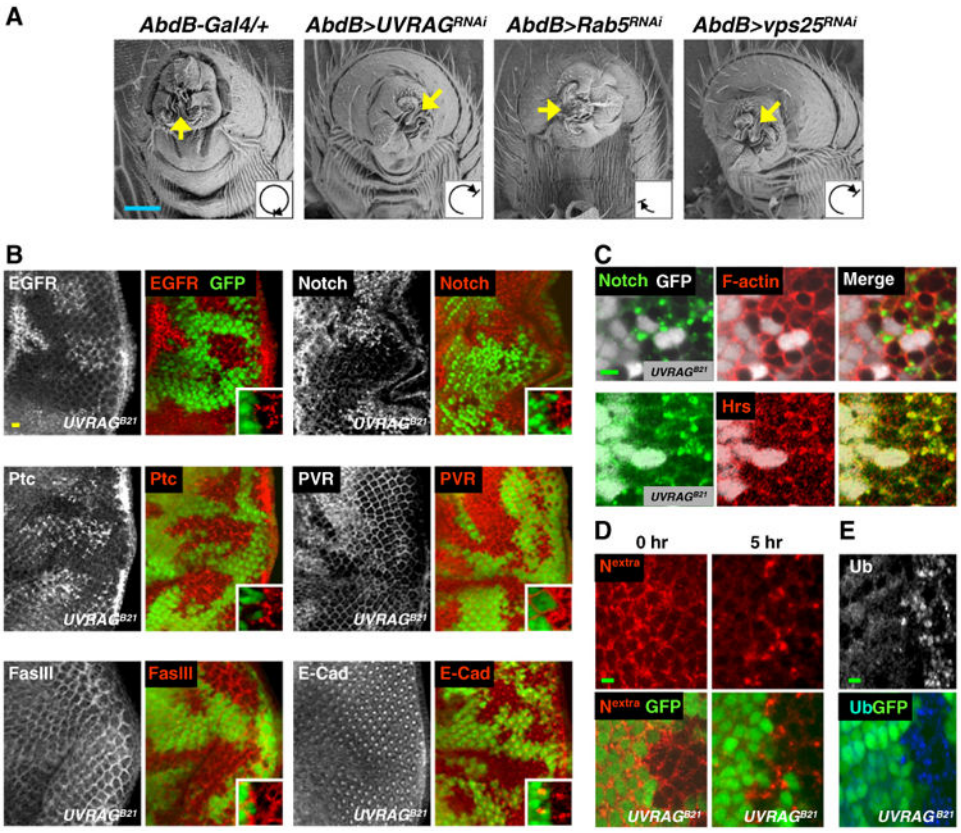

Fig. 3.

Impaired receptor endocytic degradation in UVRAG-deficient cells. (A) Ventral side views of adult male genitalia were visualized by SEM. (B, C, E) Larval eye discs containing *UVRAG* null (*UVRAG*^{B21}) clones were immunostained with antibodies against the indicated proteins and/or stained with TRITC-phalloidin (C). Inlets in (A) show the magnified images. (D) Endocytosis assay in live larval eye discs using anti-Notch extracellular domain antibody (N^{extra}, red). Scale bars: blue, 50 μm; yellow, 10 μm; green, 3 μm. Absence of GFP marks *UVRAG*^{B21} clones. Genotype: (B–E) *eyFLP/+*; *UVRAG*^{B21}*FRT40A/FRT40A UbiGFP*.

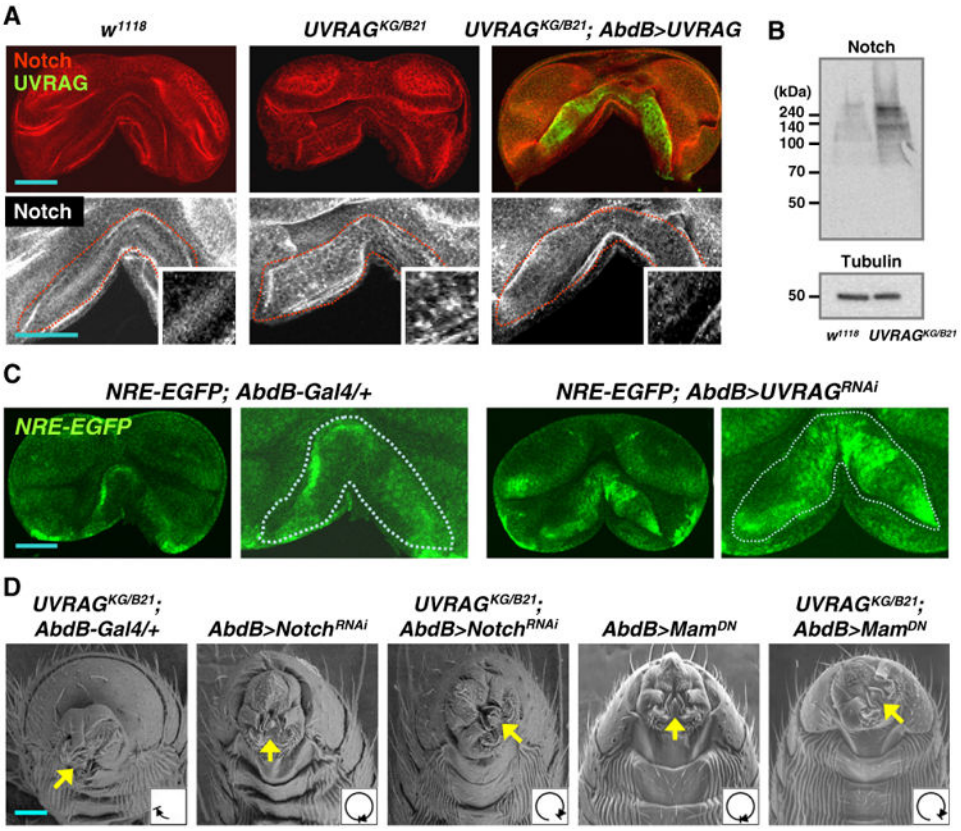

Fig. 4.

Enhanced Notch activity is associated with the impaired genitalia rotation in *UVRAG* mutants. (A) Larval genital discs were immunostained with anti-Notch (red and white in upper and lower panels, respectively) and anti-UVRAG (green in upper panels) antibodies. Lower panels show the magnified images of the upper panels. Inlets in the lower panels show the highly magnified images. Red lines in the lower panels show the *AbdB-Gal4*-specific region. (B) Immunoblot analyses using anti-Notch intracellular domain antibody in the male pupa genital discs from wild type (w^{1118}) and *UVRAG* mutant ($UVRAG^{KG/B21}$). Full-length Notch is observed around 300 kDa and cleaved Notch is observed around 120 kDa. Tubulin was used as a loading control. (C) Comparison of the Notch activity using the *NRE-EGFP* reporter in larval genital discs expressing *Gal4* control (left) or *Gal4*-driven *UVRAG* RNAi (right). Right panels show the magnified images of the left panels. White lines in the right panels show the *AbdB-Gal4*-specific region. (D) Ventral side views of the adult male genitalia from the indicated genotypes. Mam^{DN}, *Matermind-N* (Dominant negative form for transcription co-activator activity). Scale bars: 50 μm.

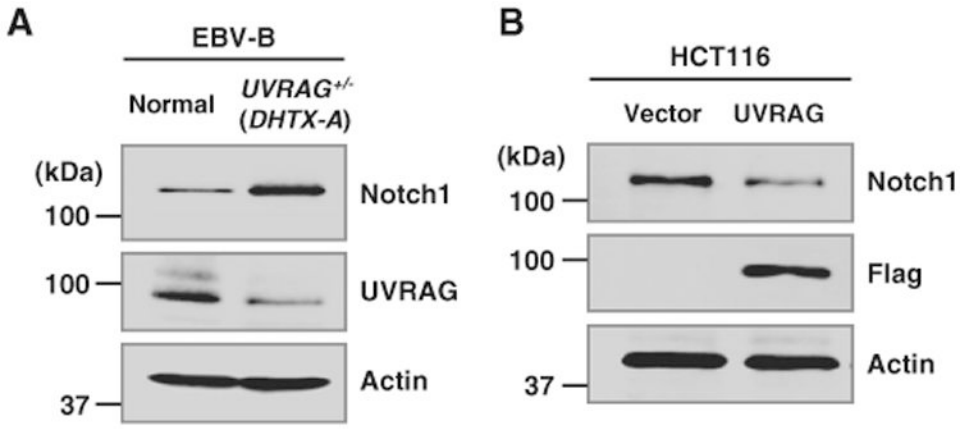

Fig. 5.

UVRAG-deficient human cells show increased Notch level. (A, B) Immunoblot analyses using anti-Notch1, anti-UVRAG or anti-Flag antibodies in EBV-B cells from a normal patient or a heterotaxy patient with monoallelic disruption of *UVRAG* (*UVRAG*$^{+/-}$; *DHTX-A*) (A) or *UVRAG*-mutated HCT116 cancer cell lines stably expressing empty vector or Flag-human UVRAG (B). Actin was used as a loading control.

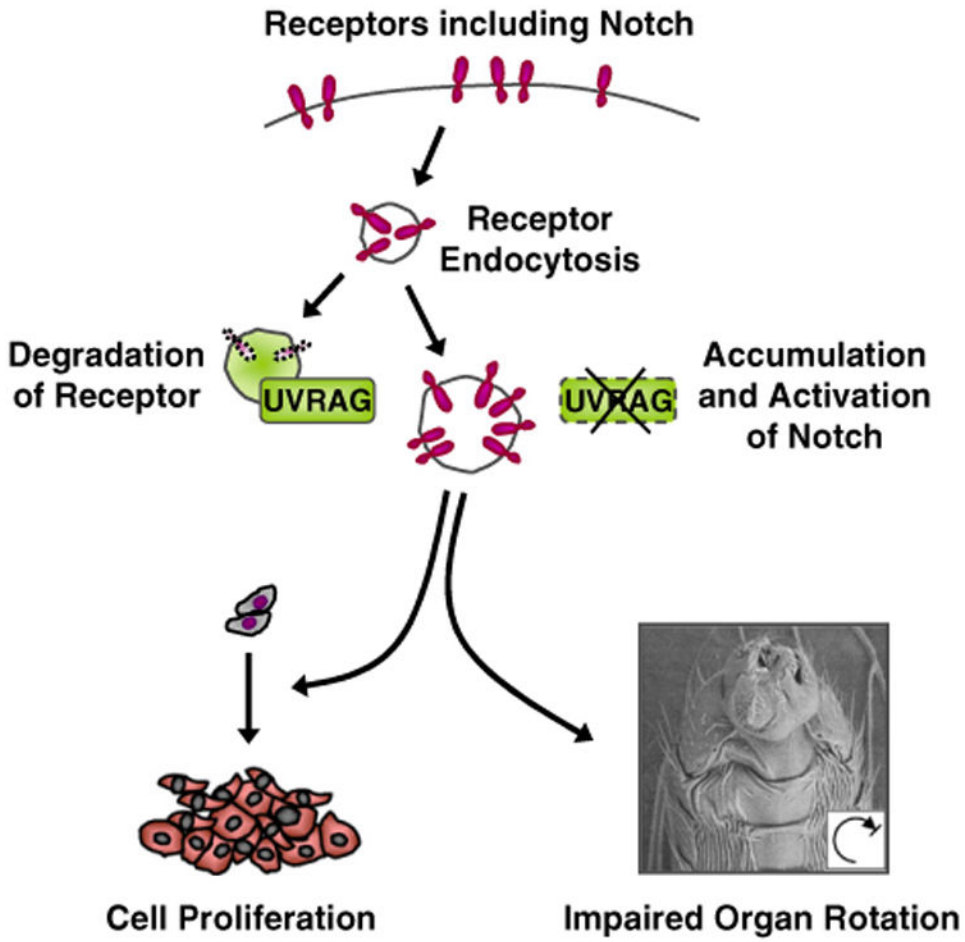

Fig. 6.

The proposed action model of UVRAG. UVRAG-mediated endocytosis promotes degradation of Notch. In the absence of UVRAG, Notch is abnormally accumulated. The accumulation and activation of Notch lead to hyper cell proliferation and failure of left–right body patterning.

Table 1

Quantification of genitalia rotation phenotypes. Numbers show the percentage of male flies with the indicated genitalia rotation phenotypes: =360° (normal), complete rotation; ≥180° (mild phenotype) and <180° (severe phenotype), incomplete rotation. The direction of rotation is all dextral. N>50 for each genotype. Interestingly, the presence of *AbdB-Gal4* itself enhances the UVRAG mutant phenotype. The reason for this is unknown. $Notch^{ICD}$, Constitutive active form; $Notch^{ECN}$, Dominant negative form for receptor interaction activity; *Mastermind-N*, Dominant negative form for transcription co-activator activity

	= 360°	**≥180°**	**<180°**
w^{1118}	100	0	0
$UVRAG^{KG04163}$	50	50	0
$UVRAG^{KG04163/B7}$	26	36	38
$UVRAG^{KG04163/B21}$	26	38	36
AbdB-Gal4/+	100	0	0
AbdB>$UVRAG^{RNAi}$	40	60	0
AbdB>$Rab5^{RNAi}$	30	25	44
AbdB>$vps25^{RNAi}$	70	30	0
AbdB>$EGFR^{RNAi}$	100	0	0
AbdB>$Notch^{RNAi}$	100	0	0
AbdB>PVR^{RNAi}	100	0	0
AbdB>Ptc^{RNAi}	100	0	0
AbdB>Notch	46	54	0
AbdB>$Notch^{ICD}$	0	0	100
AbdB>$Notch^{ECN}$	100	0	0
AbdB>Mastermind-N	100	0	0
Rescue experiments ($UVRAG^{KG04163/B21}$ background)			
AbdB-Gal4/+	0	0	100
AbdB>UVRAG	100	0	0
AbdB>$EGFR^{RNAi}$	0	0	100
AbdB>$Notch^{RNAi}$	36	44	20
AbdB>PVR^{RNAi}	0	0	100
AbdB>Ptc^{RNAi}	0	0	100
AbdB>$Notch^{ECN}$	0	0	100
AbdB>Mastermind-N	30	42	28