Endolysosomal trafficking of viral G protein-coupled receptor functions in innate immunity and control of viral oncogenesis

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The ubiquitin-proteasome system degrades viral oncoproteins and other microbial virulence factors; however, the role of endolysosomal degradation pathways in these processes is unclear. Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma, and a constitutively active viral G protein-coupled receptor (vGPCR) contributes to the pathogenesis of KSHV-induced tumors. We report that a recently discovered autophagy-related protein, Beclin 2, interacts with KSHV GPCR, facilitates its endolysosomal degradation, and inhibits vGPCR-driven oncogenic signaling. Furthermore, monoallelic loss of *Becn2* in mice accelerates the progression of vGPCR-induced lesions that resemble human Kaposi's sarcoma. Taken together, these findings indicate that Beclin 2 is a host antiviral molecule that protects against the pathogenic effects of KSHV GPCR by facilitating its endolysosomal degradation. More broadly, our data suggest a role for host endolysosomal trafficking pathways in regulating viral pathogenesis and oncogenic signaling.

endolysosomal trafficking | Beclin 2 | autophagy | viral GPCR | oncogenesis

Phagocytosis and autophagy are two processes that deliver microbes and their constituent proteins to the lysosome for degradation, thereby contributing to the clearance of pathogens and to the presentation of peptide antigens to T cells (1, 2). However, it is not known whether endocytic internalization and lysosomal targeting of virus-encoded cell-surface receptors contributes to the control of viral infection and disease.

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of AIDS-related and other forms of Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman's disease (3–5). KS is a multifocal tumor characterized by proliferating spindle cells (possibly of endothelial origin), angiogenesis, vascular slits, erythrocyte extravasation, and inflammatory cells. Proinflammatory signaling by the dominant KS cell, the spindle cell, is considered the driving force in KS lesions (6). The risk of KSHV-associated malignancies increases with increased lytic viral replication (7–9), suggesting that KSHV-induced oncogenesis may be related to the levels of expression of viral oncoproteins.

The oncogenic KSHV G protein-coupled receptor (vGPCR), encoded by the KSHV *ORF74* lytic gene, is a constitutively active chemokine receptor expressed in patients with KSHV-associated tumors (10). At least in animal studies, there are strong data that vGPCR substantially contributes to the onset and progression of KSHV-associated neoplasia in vivo (11–19). Although only a small proportion of tumor cells express vGPCR (10), they are both sufficient and necessary for KSHV-induced sarcomagenesis. The endothelial-specific expression of vGPCR (but of neither KSHV latent genes, such as *vCyclin*, *vFlip*, and *Kaposin*, nor other KSHV lytic genes, such as *vBcl-2* or *vIRF1*) or injection of murine endothelial cells stably expressing vGPCR (but not other KSHV genes, such as *vCyclin*, *vFlip*, *Kaposin*, *LANA*, *vIL-6*, *vBcl-2*, and *K1*) causes multifocal KS-like tumors in mice (15, 18). Furthermore, injection of a small number of endothelial cells expressing vGPCR increases the tumorigenic potential, in a paracrine fashion, of endothelial cells expressing other KSHV latent genes (*vCyclin* and *vFlip*), whereas eradication of the small number of vGPCR-expressing cells in established mix-cell tumors induces tumor regression (15, 18). Moreover, in a nude mouse model of KS driven by transfection of a KSHV bacterial artificial chromosome into bone marrow endothelial-lineage cells, siRNA interference (RNAi)-mediated suppression of vGPCR expression dramatically reduces angiogenesis and tumor formation (19). In addition, immunocompetent mice that transgenically express doxycycline (DOX)-inducible KSHV GPCR in endothelial cells (hereafter referred to as *ikGPCR*$^{+}$) manifest lesions that strongly resemble human Kaposi's sarcoma (16, 17). Importantly, the progression of lesions in *ikGPCR*$^{+}$ mice is reversible because DOX withdrawal leads to significant regression of vGPCR-induced lesions (17), suggesting that vGPCR-driven oncogenesis is highly dependent on sustained vGPCR expression and signaling.

Based on these previous observations in animal models regarding KSHV GPCR and oncogenesis, we developed the hypothesis that cell-intrinsic mechanisms that decrease vGPCR protein levels may function as an important host defense mechanism for controlling viral oncogenesis. Recently, we showed that the autophagy protein, Beclin 2 (but not the related autophagy protein Beclin 1) is essential for the endolysosomal degradation of certain cellular GPCRs that are regulated by GASP1 rather

Significance

We show that the autophagy-related protein Beclin 2 functions as a newly described cellular regulator of viral G protein-coupled receptors (GPCRs) and as a suppressor of viral GPCR-driven tumorigenesis. Beclin 2 functions in regulating Kaposi's sarcoma-associated herpesvirus-encoded GPCR levels, proinflammatory signaling, and oncogenic activity in mice by facilitating viral GPCR endolysosomal trafficking. This Beclin 2-dependent endolysosomal trafficking and degradation of an oncogenic viral protein may represent a broader and heretofore unappreciated role of the endolysosomal trafficking machinery in innate immunity (by defending against microbial virulence factors) and in tumor suppression (by degrading oncogenic cell surface receptors).

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than by ubiquitination and the endosomal sorting complexes required for the transport pathway (20). This function of Beclin 2, but not Beclin 1, regulates mouse brain cannabinoid receptor levels and metabolism in vivo (20). Therefore, we investigated whether Beclin 2 may play a role in the endolysosomal degradation of viral GPCRs and thereby represent an important host defense mechanism against KSHV GPCR-induced oncogenic effects. Our results demonstrate a crucial role for Beclin 2 in KSHV GPCR trafficking, proinflammatory signaling, and in vivo tumorigenicity, and thus represent a previously undescribed role for endolysosomal trafficking in innate immunity and the control of viral GPCR-driven oncogenesis.

Results

Beclin 2 Interacts with KSHV GPCR (vGPCR) and Reduces Its Protein Levels. We investigated whether Beclin family members (21) interact with vGPCR. In HEK293 cells transfected with HA-tagged vGPCR and Flag-tagged human Beclin 1 or Beclin 2, both human Beclin 1 and human Beclin 2 coimmunoprecipitated with vGPCR (Fig. 1*A*). We confirmed that endogenous Beclin 1 and Beclin 2 coimmunoprecipitated with HA-vGPCR (Fig. 1*B*). Because Beclin 2, but not Beclin 1, overexpression appeared to result in a decrease in HA-vGPCR steady-state levels (Fig. 1*A*), we examined whether there was a dose-dependent effect of Beclin 2 on vGPCR protein levels. Indeed, increasing levels of Beclin 2 expression were associated with significant decreases in levels of steady-state vGPCR expression but not of an irrelevant transfected control protein, GFP; in contrast, overexpression of Beclin 1 had no effect on steady-state levels of vGPCR (Fig. 2*A*). Moreover, siRNA knockdown of Beclin 2, but not the related autophagy protein Beclin 1 or another autophagy protein ATG7, resulted in an increase in steady-state levels of vGPCR (Fig. 2*B*), although siRNA knockdown of Beclin 2, Beclin 1, and ATG7 resulted in a comparable defect in starvation-induced autophagic flux [as measured by p62 degradation reversed by the lysosomal inhibitor bafilomycin A1 (Baf A1)] (Fig. S1). Moreover, siRNA knockdown of Beclin 2 also increased KHSV GPCR levels in body cavity lymphoma cells with lytic KSHV replication (Fig. 2*C*). Taken together, our data show that Beclin 2, but not other autophagy proteins such as Beclin 1 or ATG7, regulates cellular levels of vGPCR.

Next, we investigated whether interaction with GASP1 is required for this function of Beclin 2. Previously, we found that Beclin 2 mutants lacking amino acids 69–88 (Δ69–88) or with an I80S substitution mutation are unable to interact with GASP1 and mediate degradation of certain cellular GPCRs (20). In contrast, these two Beclin 2 mutants coimmunoprecipitated with vGPCR and decreased vGPCR steady-state levels (Fig. S2 *A* and *B*). Moreover, we rescued the increase in steady-state vGPCR levels upon Beclin 2 siRNA knockdown by expressing either wild-type siRNA-resistant Beclin 2 or mutant Δ69–88 or I80S siRNA-resistant Beclin 2 (Fig. S2 *C* and *D*). Thus, the increase in vGPCR steady-state levels with Beclin 2 knockdown is not a result of off-target siRNA effects and, unlike Beclin 2-dependent regulation of cellular GPCRs, Beclin 2-dependent regulation of vGPCR does not require its GASP1-interacting domain.

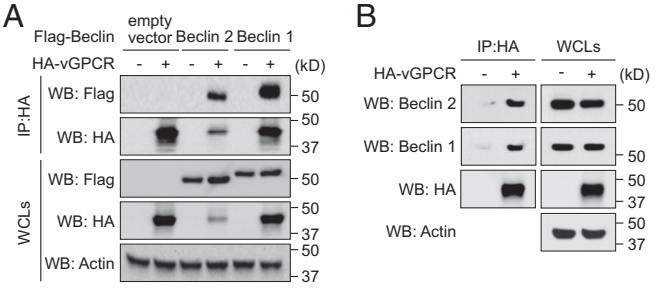

Fig. 1. Beclin 1 and Beclin 2 interact with vGPCR. (*A*) Coimmunoprecipitation of HA-vGPCR and Flag-Beclin proteins in HEK293 cells transfected with indicated plasmids. (*B*) Coimmunoprecipitation of HA-vGPCR and endogenous Beclin proteins in HEK293 cells transfected with indicated plasmids. For *A* and *B*, an anti-HA antibody was used to immunoprecipitate HA-vGPCR and similar results were observed in at least three independent experiments. WB, Western blot.

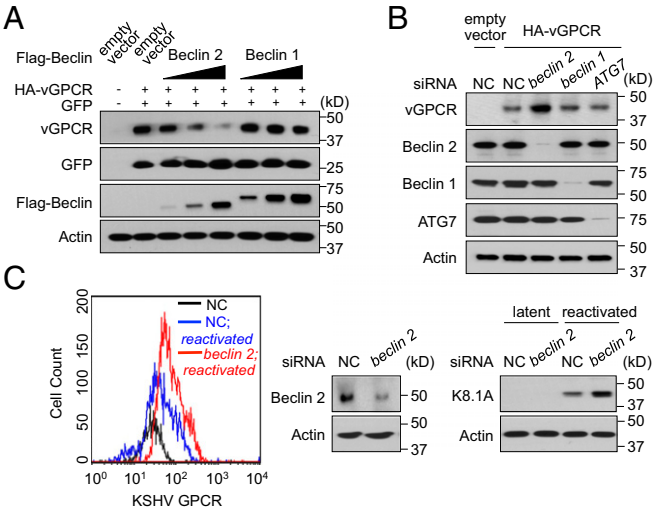

Fig. 2. Beclin 2, but not Beclin 1, reduces vGPCR protein levels. (*A*) Western blot detection of vGPCR, Beclin 2, Beclin 1, and the control GFP in HEK293 cells transfected with indicated plasmids. Three different doses of Beclin plasmids were used in transfection, and the total amount of transfected DNA was balanced with empty vector for all groups. (*B*) Western blot detection of vGPCR, endogenous Beclin 2, Beclin 1, ATG7, and actin in HEK293 cells expressing HA-tagged vGPCR and treated with siRNA targeting indicated gene. (*C*) Effects of Beclin 2 knockdown on vGPCR protein levels in KSHV-lytically infected body cavity lymphoma cells. Ten-thousand cells were analyzed per group by flow cytometry (*Left*). In an identical experiment, the *Center* panel shows Western blot analysis of endogenous Beclin 2, and the *Right* panel shows Western blot analysis of KSHV envelope glycoprotein K8.1A, confirming reactivation of KSHV lytic replication. For *A*–*C*, similar results were observed in at least three independent experiments. See also Fig. S1 and Fig. S2. NC, nontargeting control siRNA.

Beclin 2 Regulates vGPCR Protein Levels Through a Lysosomal Degradation Pathway. The Beclin 2-dependent decrease in HA-vGPCR expression was partially reversed by treatment with the lysosomal inhibitor, Baf A1 (Fig. 3*A*), suggesting that Beclin 2 may promote the degradation of KSHV GPCR through a lysosomal-dependent (but autophagy-independent) pathway. To confirm these findings using an independent approach (Fig. S3*A*), we followed the fate of fluorescently labeled surface HA-vGPCR (at 4 °C) at serial time points after internalization (incubation at 37 °C) in the presence or absence of enforced Beclin 2 expression and in the presence or absence of Baf A1. By 90 min after internalization, the percentage of cells expressing vGPCR was significantly less when cotransfected with Beclin 2 versus empty vector control (Fig. 3 *B* and *C*); this number dropped to almost 15% in the Beclin 2-transfected cells, but remained at ∼70% in the vector-transfected cells (Fig. 3*B*). This decrease in vGPCR$^+$ cells upon cotransfection with Beclin 2 was completely blocked by treatment with Baf A1 (Fig. 3*C*), confirming that Beclin 2 promotes the endolyososomal degradation of vGPCR. To confirm that endogenous Beclin 2 regulates the fate of internalized vGPCR, we compared the percentage of vGPCR$^+$ cells treated with noncoding control or *beclin 2* siRNA (Fig. S4*B*) at 45 min and 180 min after receptor internalization. At 45 min, no differences were observed and vGPCR was predominantly colocalized with the endosomal marker, early endosome antigen 1 (EEA1) (Fig. S3*C*); in contrast, at 180 min, very few (∼10%) vGPCR$^+$ cells were observed in the nontargeting control siRNAs, whereas ∼70% cells were vGPCR$^+$ in the *beclin 2* siRNA-treated group

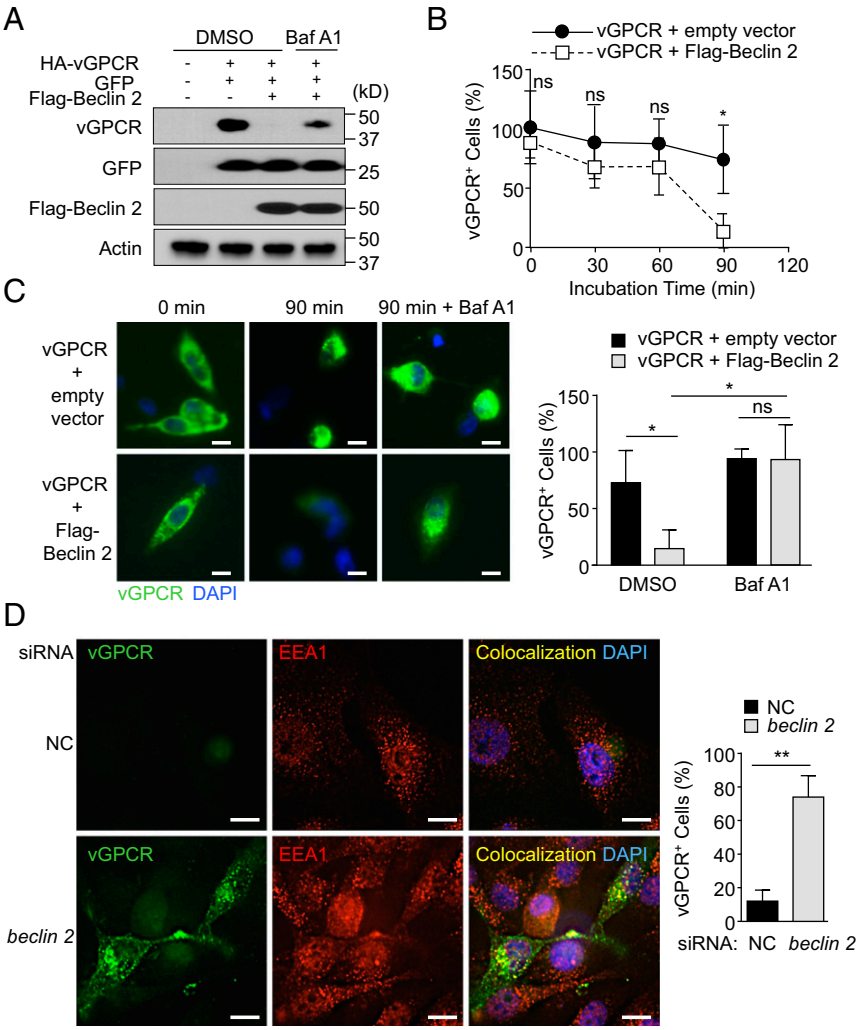

Fig. 3. Beclin 2 regulates vGPCR protein levels through a lysosomal degradation pathway. (*A*) Western blot detection of vGPCR, Beclin 2, and the control GFP in HEK293 cells transfected with indicated plasmids for 24 h and treated with DMSO or Baf A1 (100 nM) for 10 h. (*B* and *C*) Effects of Beclin 2 ectopic expression on lysosomal-dependent degradation of internalized cell surface-labeled vGPCR in HeLa cells. Antibody pulse-labeling and immunofluorescence microscopy was performed as indicated in Fig. S3A. (*B*) Kinetics of vGPCR turnover as assessed by quantitation of percentage of vGPCR$^+$ cells at serial time points during incubation at 37 °C. (*C*) Representative immunofluorescence images and quantitation of vGPCR$^+$ HeLa cells transfected with indicated plasmids and treated with DMSO or 100 nM Baf A1 after 90-min internalization at 37 °C. The relative percentage of cells containing vGPCR puncta was calculated in reference to 0 min (*Right*). (*D*) Effects of Beclin 2 knockdown on degradation of internalized cell surface-labeled vGPCR. HeLa cells were treated with indicated siRNA, transfected with a plasmid expressing HA-tagged vGPCR, and antibody pulse-labeling and immunofluorescence microscopy performed as indicated in Fig. S3A. Representative images of HA-vGPCR and EEA1 (endosomal marker) colocalization (*Left*) and quantitation of vGPCR$^+$ cells (*Right*) after 180-min internalization at 37 °C. See Fig. S3C for data after 45-min internalization at 37 °C. (Scale bars, 10 μm.) Values represent mean ± SD of triplicate samples (>100 cells analyzed per sample). For *A–D*, similar results were observed in three independent experiments. *$P < 0.05$; **$P < 0.01$; ns, not significant; *t* test.

(Fig. 3*D*). We conclude that increased Beclin 2 expression facilitates the endolysosomal degradation of vGPCR, whereas Beclin 2 knockdown delays intracellular vGPCR degradation.

Beclin 2 Suppresses vGPCR-Induced Oncogenic Signaling. We next investigated whether Beclin 2-dependent regulation of KSHV GPCR levels affects its signaling functions (16, 22–24), including activation of the NF-κB transcription factor and the NF-κB–dependent, proinflammatory cytokine IL-6, which is protumorigenic in KSHV-induced tumors (25–29). Using NF-κB and IL-6 promoter luciferase reporter assays, we found that enforced Beclin 2, but not Beclin 1, expression inhibits vGPCR-induced NF-κB and IL-6 activation in a dose-dependent manner (Fig. 4 *A* and *B*). This suppression of vGPCR-induced NF-κB and IL-6 activation was reversed by the lysosomal inhibitor Baf A1 (Fig. 4 *C* and *D*). In addition, expression of Beclin 2 mutants (Δ69–88 and I80S) that do not interact with GASP1 (20), suppressed vGPCR-induced NF-κB and IL-6 activation as effectively as wild-type Beclin 2 (Fig. S4 *A* and *B*). Moreover, Beclin 2 enforced expression did not suppress TNF-α–induced NF-κB activation (Fig. S4 *C* and *D*), suggesting the regulation of vGPCR signaling by Beclin 2 is not a result of nonspecific global suppression of NF-κB activation.

We also found that knockdown of Beclin 2, but not of Beclin 1 or ATG7, enhanced vGPCR-mediated NF-κB activation (Fig. 4*E* and Fig. S4*E*) and IL-6 activation (Fig. 4*F* and Fig. S4*F*), and this increase was reversed by cotransfection with either wild-type or GASP1 binding-defective Beclin 2 siRNA-resistant mutants (Fig. 4 *G* and *H* and Fig. S4 *G* and *H*). Taken together, these data indicate that Beclin 2 regulates vGPCR-induced NF-κB and IL-6 signaling in a lysosomal-dependent (but autophagy-independent) manner, which involves facilitating the endolysosomal degradation of vGPCR. Moreover, this function of Beclin 2 does not require its interaction with GASP1.

Beclin 2 Suppresses KSHV GPCR-Driven Oncogenesis in Vivo. Given our observation that Beclin 2 regulates vGPCR-induced oncogenic signaling in vitro, we investigated whether Beclin 2 plays a role in regulating vGPCR-induced oncogenesis in vivo. We used a previously established mouse model (referred to herein as *ikGPCR*$^+$ mice) in which DOX-inducible expression of KSHV GPCR causes lesions in mice that strongly resemble human cutaneous KS (16, 17). We crossed *ikGPCR*$^+$ mice with previously described *Becn2*$^{+/-}$ mice that are deficient in autophagy and the degradation of certain cellular GPCRs (20) and with *Becn1*$^{+/-}$ mice that are deficient in autophagy (30), but not in the degradation of cellular GPCRs (20). Compared with *ikGPCR*$^+$;*Becn2*$^{+/+}$ littermates, *ikGPCR*$^+$;*Becn2*$^{+/-}$ littermates had a significantly earlier onset of detectable skin lesions following the initiation of DOX administration in the drinking water (Fig. 5*A*). This result was not caused by increased water intake (Fig. S5*A*) or increased serum DOX levels in the *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice (Fig. S5*B*).

Both *ikGPCR*$^+$;*Becn2*$^{+/+}$ and *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice manifested the typical cutaneous lesions previously described in *ikGPCR*$^+$ mice (with vascular and spindle cell proliferation and an admixture of inflammatory cells), most prominent on the tail, ear, and other

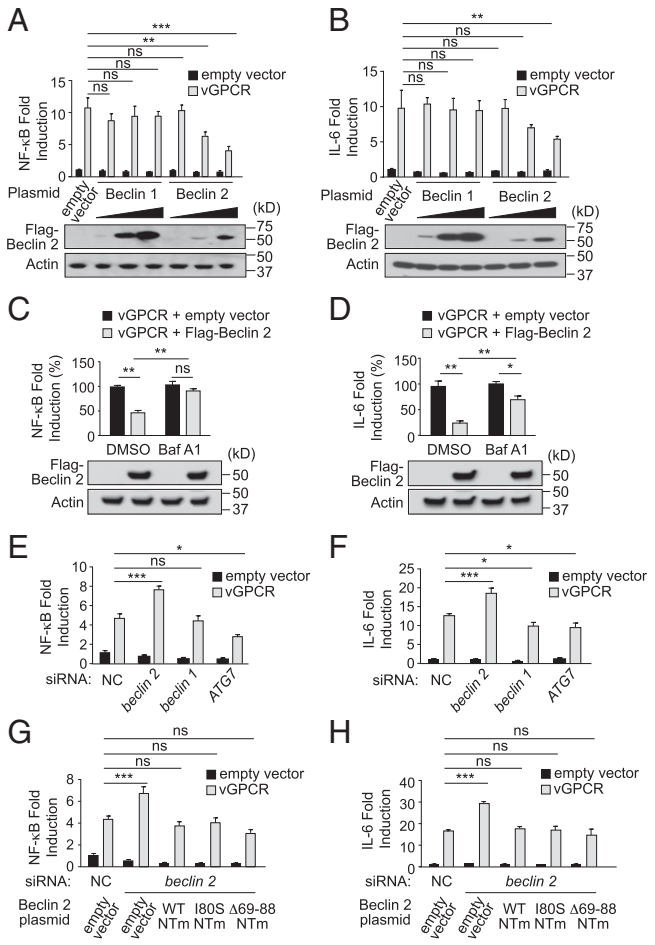

Fig. 4. Beclin 2 suppresses vGPCR-induced oncogenic signaling. (*A* and *B*) Effects of Beclin 1 and Beclin 2 ectopic expression on NF-κB (*A*) and IL-6 (*B*) promoter activity in HEK293 cells. Three different doses of Beclin plasmids were used in transfection, and the total amount of plasmids was balanced with empty vector for all groups. Levels of Flag-Beclin 2 and Flag-Beclin 1 in whole-cell lysates were determined by Western blot analysis. (*C* and *D*) Beclin 2 ectopic expression regulates NF-κB (*C*) and IL-6 (*D*) promoter activity through a lysosomal-dependent pathway. HEK293 cells were treated with either DMSO or 100 nM Baf A1 for 10 h. Levels of Flag-Beclin 2 in whole-cell lysates were determined by Western blot analysis. (*E* and *F*) Effects of Beclin 2, Beclin 1, and ATG7 knockdown on NF-κB (*E*) and IL-6 (*F*) promoter activity. HEK293 cells were treated with indicated siRNAs for 48 h, and then transfected with a plasmid expressing HA-vGPCR. See Fig. S4 *E* and *F* for Western blot showing knockdown of endogenous Beclin 2, Beclin 1, and ATG7 in these cells. (*G* and *H*) Rescue of effects of Beclin 2 knockdown on NF-κB (*G*) and IL-6 (*H*) promoter activity with siRNA-resistant Beclin 2 expression constructs. HEK293 cells were treated with indicated siRNAs for 48 h, and then transfected with empty vector or plasmids expressing siRNA-resistant (NTm) wild-type Beclin 2, or siRNA-resistant Beclin 2 mutants (I80S and Δ69–88) that are unable to interact with GASP1 (20). See Fig. S4 *G* and *H* for Western blot analysis showing knockdown of endogenous Beclin 2 and reconstitution of Beclin 2 expression in these cells. For *A*–*H*, bars represent mean ± SD of triplicate samples and similar results were observed in three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant; one-way ANOVA with Dunnett method. See also Fig. S4.

exposed skin regions. However, at the same time period after DOX treatment, the lesions in the *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice were more numerous and larger than those observed in *ikGPCR*$^+$;*Becn2*$^{+/+}$ mice at the macroscopic level (see representative photos in Fig. 5*B*) and they showed a higher density of lesional cells and more inflammatory cells upon histopathological examination (Fig. 5*C* and Fig. S6). Moreover, staining with an antibody against the endothelial cell marker, CD34, revealed a significant increase in CD34$^+$ spindle-shaped cells (Fig. 5*C*), one of the hallmark features of human KS. Immunostaining with an antibody against the vGPCR transgenic protein also revealed higher levels of vGPCR immunostaining in lesions of *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice compared with *ikGPCR*$^+$;*Becn2*$^{+/+}$ mice (see representative photomicrograph in Fig. 5*D*).

Consistent with the earlier onset and increased severity of Kaposi's sarcoma-like skin lesions in the *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice compared with the *ikGPCR*$^+$;*Becn2*$^{+/+}$ mice, *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice had significantly shorter survival than *ikGPCR*$^+$;*Becn2*$^{+/+}$ littermates (Fig. 5*E*). Mice were killed when they became visibly moribund with an inability to ambulate and manifested respiratory distress. Even though all mice were moribund at the time of autopsy, the *ikGPCR*$^+$;*Becn2*$^{+/-}$ group had larger peritoneal serosanguinous effusions, more extensive diffuse lymphedema, and more visible pulmonary vascular lesions. Microscopically, upon random lung sectioning, a higher percentage of *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice had pathological evidence of pulmonary hemorrhagic KS than *ikGPCR*$^+$;*Becn2*$^{+/+}$ (37 of 43 mice vs. 23 of 36 mice; $P < 0.05$; χ^2 test). Similar to human pulmonary KS (31), pulmonary lesions displayed slit-like vascular spaces and extensive erythrocyte extravasation, and tended to be more extensive in the *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice (Fig. 5*F*). *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice also had a marked increase in serum levels of IL-6 at the time of death (Fig. 6*A*) and at 2 and 4 wk after DOX treatment (Fig. 6*B*). The variability of IL-6 production among mice is likely a result of individual variation instead of leaky expression from the DOX-responsive *ikGPCR* allele, as the serum levels of IL-6 in untreated *ikGPCR*$^+$;*Becn2*$^{+/+}$ and *ikGPCR*$^+$;*Becn2*$^{+/-}$ littermate mice were undetectable. The levels of serum IL-6 elevation in *ikGPCR*$^+$;*Becn2*$^{+/-}$ mice inversely correlated with duration of survival (Fig. S7), consistent with data from previous animal models and human studies, suggesting that IL-6 is an important pathogenic factor in KS-like disease (25–29, 32).

Thus, allelic loss of *beclin 2* significantly exacerbates KSHV GPCR-induced protumorigenic signaling and KSHV GPCR-induced neoplastic lesions in vivo. These effects are unlikely to be related to autophagy, because *ikGPCR*$^+$;*Becn1*$^{+/-}$ mice did not have accelerated onset of lesions, earlier mortality, or increased IL-6 production compared with littermate *ikGPCR*$^+$;*Becn1*$^{+/+}$ control mice (Fig. S8). Rather, taken together with our in vitro findings, they most likely reflect a role for Beclin 2 in promoting the endolysosomal degradation of KSHV GPCR and, thereby, in blocking its protumorigenic signaling effects.

Discussion

Our findings demonstrate a crucial role for the endolysosomal degradation of a virally encoded cell-surface receptor in the suppression of proinflammatory signaling and neoplastic disease driven by a viral oncogenic protein. Overexpression of Beclin 2 (but not the related autophagy protein Beclin 1) results in accelerated degradation of vGPCR and decreased proinflammatory signaling that is blocked by lysosomal inhibition, whereas knockdown of Beclin 2 (but not the related autophagy protein Beclin 1) results in delayed degradation of vGPCR and increased proinflammatory signaling. Moreover, allelic loss of *Becn2*, but not of *Becn1*, in mice results in accelerated progression and enhanced severity of vGPCR-driven tumorigenesis, as well as increased IL-6 signaling. Therefore, we propose that this Beclin 2-dependent endolysosomal trafficking and degradation of a KSHV oncogenic protein may represent a broader and heretofore unappreciated role of the endolysosomal trafficking machinery in innate immunity (by functioning as a defense against microbial virulence factors) and in tumor suppression (by degrading oncogenic cell surface receptors).

Considerable advances have been made in defining how oncogenic viral factors (e.g., vGPCR, vFLIP, vCyclin, and vIL-6) contribute to KSHV-induced oncogenesis (33, 34), and how onocogenic herpesviruses evade or manipulate host defense pathways (including autophagy) (35, 36). However, it is still largely unclear what mechanisms the host uses to successfully defend

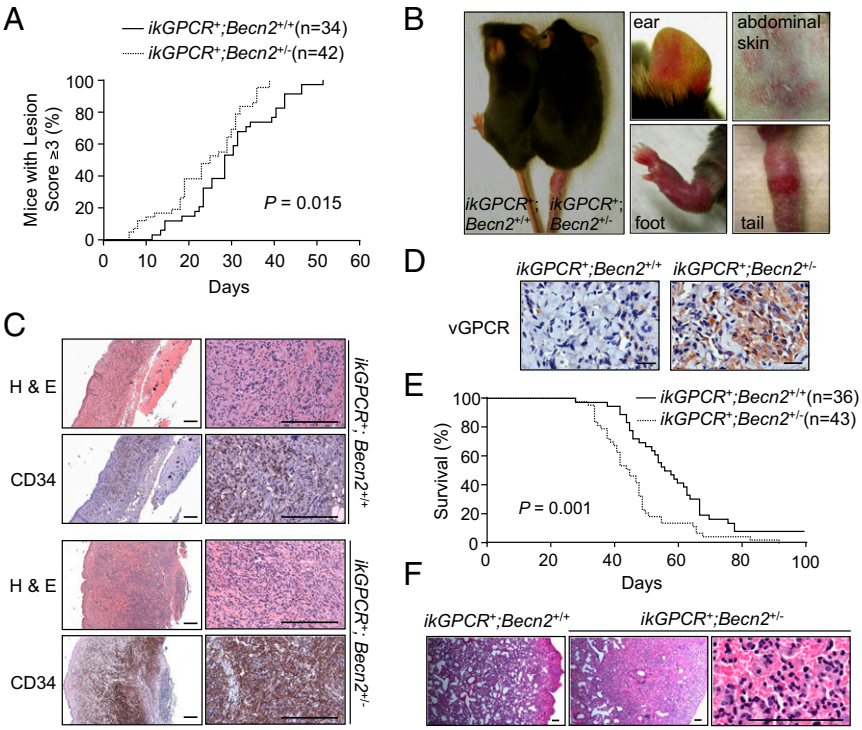

Fig. 5. Monoallelic loss of *Becn2* in mice accelerates KSHV GPCR-driven oncogenesis. (*A*) Lesion incidence in DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ mice. No lesions were observed in mice of either genotype in the absence of DOX treatment (Table S1). Statistical significance assessed by log-rank test; number of mice per genotype and *P* value indicated in graph. (*B*) Representative photomicrographs of a pair of *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ littermates after 40 d of DOX treatment. Images of ear, foot, abdominal skin, and tail (*Right*) are higher-power magnification of the *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ mouse shown (*Left*). (*C*) Representative photomicrographs of H&E and anti-CD34–stained sections of abdominal skin tumor specimens from DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ mice. (Scale bars, 200 μm.) (*D*) Representative images of anti-vGPCR immunohistochemistry staining of abdominal skin tumor specimens from DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ mice. (Scale bars, 20 μm.) (*E*) Kaplan–Meier curves of survival time of DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ littermates. Statistical significance assessed by log-rank test; number of mice per genotype indicated in graph. (*F*) Representative photomicrographs of H&E-stained sections of lung specimens from DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ mice. (Scale bars, 100 μm.) See also Figs. S5, S6, and S8, and Table S1.

against oncogenesis driven by γ-herpesviruses. Although immune status (such as HIV-related or iatrogenic immunosuppression) and ethnicity (such as in classic KS and endemic KS) are important determinants of risk of KSHV-associated malignancies (34, 37), other unexplained factors likely play a role in determining the incidence and prevalence of KS in at-risk populations. Our findings in mice (i.e., the acceleration of disease in vGPCR transgenic mice with allelic loss of *Becn2*) lead us to speculate that genetic variations in *BECN2* (or genes encoding other proteins that may regulate the trafficking of vGPCR) contribute to individual susceptibility to KSHV-associated malignancies in humans.

The mechanism by which Beclin 2 protects against KSHV GPCR-induced oncogenesis likely relates to a role in reducing vGPCR protein levels in endothelial cells harboring the transgene and subsequent reduction of IL-6 protumorigenic signaling. Our in vitro studies demonstrate a lysosomal-dependent role for Beclin 2 in reducing vGPCR levels and vGPCR-induction of IL-6 signaling, and our in vivo studies show enhanced vGPCR expression and increased IL-6 production in mice with allelic loss of *Becn2*. Moreover, the magnitude of IL-6 elevation in *Becn2*$^{+/-}$ mice inversely correlates with survival time. Several lines of evidence suggest that IL-6 is a key pathogenic factor in KSHV-associated malignancies (25–29, 32). In humans, an IL-6 promoter polymorphism (G-174C), which leads to increased IL-6 expression (38, 39), is strongly associated with different types of KS, including those that occur in AIDS patients (epidemic KS) (28), renal transplant recipients (iatrogenic KS) (40), and a familiar cluster of classic KS (41). In mice, genetic deletion of IL-6 ablates KSHV-associated multicentric Castleman's disease (26). Although our studies do not prove a causal relationship between increased IL-6 production and accelerated tumorigenesis and mortality in *Becn2*$^{+/-}$ mice, our observations are consistent with the paradigm that IL-6 is a key regulator of KSHV pathogenesis and provide definitive evidence that Beclin 2 regulates KSHV GPCR-induced IL-6 levels in mice.

In conclusion, our findings indicate that the endolysosomal degradation of viral (and potentially other microbial) virulence factors may serve as an important host antipathogen defense mechanism. Previous studies have shown that the canonical autophagy machinery can function in antibacterial and antiviral host defense by delivering intracellular pathogens or components of intracellular pathogens to the lysosome for degradation (2, 36). Moreover, Beclin 1 and the autophagy pathway has been proposed to function in controlling viral oncogenesis in at least two contexts: monoallelic deletion of *Becn1* accelerates neoplastic lesions in the livers of mice that transgenically express hepatitis B envelope protein autophagy (30) and decreased autophagic degradation of a microRNA (miR-224) is postulated to contribute to hepatitis B virus-associated hepatocellular carcinoma in mice and in humans (42). Our findings suggest that independently of the autophagy machinery, the delivery of viral oncoproteins to the lysosome for degradation (via an endolysosomal trafficking route) may play a crucial role in innate immunity.

Materials and Methods

See *SI Materials and Methods* for a detailed description.

Cell Lines and Mouse Strains. HEK293 and HeLa cell lines were obtained from the American Type Culture Collection and KSHV latently infected body

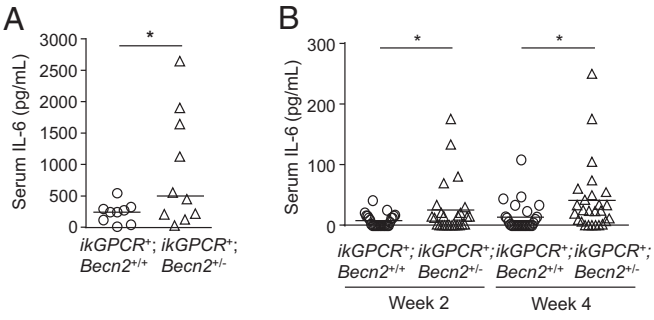

Fig. 6. Monoallelic loss of *Becn2* in mice increases KSHV GPCR-driven IL-6 production. Serum IL-6 levels of DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ (n = 9) and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ (n = 10) mice on the day of autopsy (*A*) or of DOX-treated *ikGPCR*$^{+}$;*Becn2*$^{+/+}$ (n = 27) and *ikGPCR*$^{+}$;*Becn2*$^{+/-}$ (n = 26) mice at indicated time points after DOX treatment (*B*). Solid lines represent the sample median of each group. *P < 0.05, t test. See also Figs. S7 and S8.

cavity-based lymphoma (Bcbl-1) cells that carry the tetracycline-inducible viral replication and transcription activator (Rta) allele (Bcbl-1.TREx-Rta) were a gift from Jae U. Jung, Keck School of Medicine, University of Southern California, Los Angeles (43). Mouse strains used in this study have been previously described, including *Becn1*$^{+/-}$ (30), *Becn2*$^{+/-}$ (20), and the transgenic mouse strain that expresses tetracycline-inducible KSHV GPCR (*ikGPCR*$^{+}$), known as *iORF74* mice in previous studies (16, 17). All animal protocols were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Detailed information on cell culture conditions, mouse breeding and genotyping, animal experiments, and histopathological analyses of animal tissues is provided in *SI Materials and Methods*.

Antibodies, Chemical Reagents, Plasmids, and siRNAs. See *SI Materials and Methods* for details.

Western Blotting and Coimmunoprecipitation Studies. See *SI Materials and Methods* for details.

Luciferase Reporter Assays. NF-κB and IL-6 promoter activity was measured by performing luciferase reporter assays as described in the *SI Materials and Methods*.

Microscopy Studies. For antibody-pulse labeling, HeLa cells expressing HA-vGPCR were transfected with nontargeting control siRNAs or siRNA targeting beclin 2, incubated with an anti-HA antibody for 45 min on ice, and then incubated for 45–180 min at 37 °C to allow vGPCR-antibody complex internalization. All imaging was performed using a Zeiss AxioImager Z2 microscope, and *z*-stack images were deconvolved with AutoDeBlur, and analyzed with Imaris v7.4.0 (Bitplane). See *SI Materials and Methods* for details.

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