HRAS-driven cancer cells are vulnerable to TRPML1 inhibition

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Abstract

By serving as intermediaries between cellular metabolism and the bioenergetic demands of proliferation, endolysosomes allow cancer cells to thrive under normally detrimental conditions. Here, we show that an endolysosomal TRP channel, TRPML1, is necessary for the proliferation of cancer cells that bear activating mutations in HRAS. Expression of MCOLN1, which encodes TRPML1, is significantly elevated in HRAS-positive tumors and inversely correlated with patient prognosis. Concordantly, MCOLN1 knockdown or TRPML1 inhibition selectively reduces the proliferation of cancer cells that express oncogenic, but not wild-type, HRAS. Mechanistically, TRPML1 maintains oncogenic HRAS in signaling-competent nanoclusters at the plasma membrane by mediating cholesterol de-esterification and transport. TRPML1 inhibition disrupts the distribution and levels of cholesterol and thereby attenuates HRAS nanoclustering and plasma membrane abundance, ERK phosphorylation, and cell proliferation. These findings reveal a selective vulnerability of HRAS-driven cancers to TRPML1 inhibition, which may be leveraged as an actionable therapeutic strategy.

Keywords cancer; cholesterol; endolysosomes; HRAS; TRPML1

Introduction

Mutated RAS oncogenes, which represent leading causes of cancer [1], encode constitutively active small G proteins that are currently beyond the reach of direct pharmacological inhibition. Although inhibition of downstream effectors—BRAF, MEK, or ERK—has been met with some success, efficacy of these strategies is limited by factors such as ubiquity of MEK–ERK signaling, propensity for acquired resistance, and myriad feedback loops associated with unremitting RAS activity [1]. Pharmacological inhibition of BRAF, for example, induces paradoxical activation of RAS–ERK signaling and the undesirable potentiation of cell proliferation [2]. Alternatively, development of resistance to RAF or MEK inhibition due to somatic mutations and/or gene amplifications can reinitiate ERK activation and tumorigenesis [3].

An approach to overcome these obstacles involves the identification and disruption of ancillary cellular processes that are selectively upregulated in RAS-driven cancers. This strategy may reveal potential vulnerabilities that can be exploited to mitigate oncogenesis. For example, molecular mechanisms that permit cancer-specific reorganization of cellular metabolism constitute pathways that could be targeted to deter tumorigenesis with exquisite sensitivity and specificity [4–6]. In this context, components of the autophagic and endolysosomal system represent actionable targets [7–11]. Indeed, arresting autophagy and lysosomal degradation via dissipation of the endolysosomal pH gradient using chloroquine is beneficial in some preclinical cancer models, although it is not clear whether the sensitivity to chloroquine correlates with RAS mutations [12,13].

In order to prevent unintended potential side effects of blanketed endolysosomal ablation, we reasoned that a cogent strategy to mitigate tumorigenesis would involve the prior determination of the endolysosomal proteins that contribute to disease. To this end, we examined the patterns of endolysosomal gene expression in HRAS-driven tumors. We found that tumors with oncogenic HRAS mutations exhibit a gene expression signature that reflects increased endolysosomal biogenesis via the Mitf/Tfe3/Tfeb-family of transcription factors [14–17]. Importantly, the gene encoding an endolysosomal cation channel, MCOLN1, was a core element of this transcriptional program. We found that oncogenic HRAS-expressing

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cancer cells were vulnerable to TRPML1 inhibition or MCOLN1 knockdown. Investigation of the underlying mechanisms revealed a role for TRPML1 in the maintenance of plasma membrane cholesterol levels. The mislocalization of plasma membrane cholesterol following inhibition of TRPML1 deterred HRAS<sup>G12V</sup>-driven ERK activation. These studies underscore the utility of a systems approach to identify disease-specific endolysosomal proteins, and raise the possibility that targeting the function of TRPML1 could limit the growth of cancers driven by oncogenic HRAS.

Results

The endolysosomal gene expression signature in tumors bearing oncogenic HRAS mutations suggests a role for MCOLN1

Cancers of the bladder, head and neck region, and thyroid exhibit a propensity for activating mutations in HRAS [18–20]. Our examination of TCGA datasets revealed that cancers with the highest fraction of HRAS mutations at codons 12, 13, 61, and 117 were bladder urothelial carcinoma (BLCA), head and neck squamous cell carcinoma (HNSC), and thyroid carcinoma (THCA) (~60% of patients with oncogenic HRAS mutations presented with one of these 3 diseases). We asked whether gene expression patterns indicative of endolysosomal biogenesis are apparent in these HRAS-driven tumors. We focused our attention on 210 genes that encode a broad spectrum of endolysosomal proteins including hydrolytic enzymes, subunits of the V-ATPase complex, ion channels, transporters, regulators of vesicle trafficking, and components of autophagy/mTOR-related metabolic processes. RNA-seq analyses of HNSC, BLCA, and THCA tumors and matched normal tissues revealed a gene expression signature comprised of 72 significantly upregulated genes, 61 significantly downregulated genes, and 77 genes whose expression did not change significantly (Fig 1A). Upregulated genes included MCOLN1, which encodes a PI(3,5)P<sub>2</sub>-activated endolysosomal Ca<sup>2+</sup> channel called TRPML1 [21]. Also upregulated was VAC14, whose protein product is part of an endosomal complex required for PI(3,5)P<sub>2</sub> synthesis (Fig 1A, inset) [21,22]. Mirroring the increase in VAC14 expression, expression of MTM1, which encodes a lipid phosphatase that breaks down PI(3,5)P<sub>2</sub> (Fig 1A, inset) [23], was decreased in HRAS tumors (Fig 1A; yellow circle). This, HRAS transformed tumors demonstrate a juxtaposition of elevated MCOLN1 expression and a possible shift in the dynamics of PI(3)P–PI(3,5)P<sub>2</sub> interconversion toward synthesis of PI(3,5)P<sub>2</sub>—the endosomal phosphoinositide that activates TRPML1.

Analysis of endolysosomal gene expression reveals underlying regulatory mechanisms and implicates MCOLN1 as an actionable hub in HRAS tumors

Unsupervised hierarchical clustering of the pairwise correlations of gene expression revealed four major clusters of coregulated genes (Fig 1B and Appendix Fig S1). Average Z-scores indicated increased expression of genes belonging to clusters 1 and 3 and repression of genes belonging to cluster 4 (Fig 1C). VAC14 and MCOLN1 belonged to clusters 1 and 3, respectively, whereas MTM1 belonged to cluster 4. These data suggest coordinated patterns of endolysosomal gene expression in tumors bearing oncogenic mutations in HRAS. This insight led us to focus on the Mitf/Tfe3/Tfeb transcription factors, which drive <i>en masse</i> expression of endolysosomal genes that belong to the “Coordinated Lyosomal Expression and Regulation” (CLEAR) family [14–17]. Gene set enrichment analysis (GSEA) [24] of the endolysosomal gene expression signature revealed enriched expression of CLEAR targets in HRAS-driven tumors (Fig 1D). In agreement with these bioinformatic analyses, RT-qPCR revealed that expression of <i>MITF</i>, <i>TFE3</i>, and <i>TFE3</i> is elevated in oncogenic HRAS-positive HN31 oral cancer cells [25] in comparison with HN31 cells stably expressing an shRNA against HRAS (Fig 1E). Using previously validated antibodies [26], we found that protein levels of Tfeb were also elevated in HN31 cells compared to the variants stably expressing HRAS shRNA (Fig 1F). Furthermore, expression of the CLEAR target genes—MCOLN1, CTSA, CTSD, VAC14, LAMP1, and GBA—was significantly elevated in an HRAS-dependent manner (Fig 1G). Concordantly, knockdown of <i/MITF</i> or <i>TFEB</i> decreased the...
Figure 1.

TRPML1 allows cancer cell proliferation
expression of these endolysosomal genes (Fig EV1A). These data demonstrate HRAS-dependent upregulation endolysosomal genes belonging to the CLEAR network.

Biological networks are comprised of sparsely distributed hubs that exhibit disproportionately higher connectivity than other nodes [27]. This architecture bestows robustness to networks since stochastic failures naturally exhibit higher probabilities of impacting the numerous outlying nodes rather than the relatively few hubs. However, focused attacks on hubs can dismantle wide swaths of the network. Based on this understanding, we reasoned that targeting hubs of the endolysosomal gene expression network might impact the growth of oncogenic HRAS-induced tumors. To test this idea, we first constructed a network based on pairwise correlations of endolysosomal gene expression in tumors bearing oncogenic HRAS mutations. Measures of network centrality revealed that genes belonging to the CLEAR network had higher overall centrality in the network (Fig EV1B). Gene set enrichment analysis performed on the endolysosomal gene set ranked on the basis of centrality scores also pointed to enrichment of CLEAR targets in the centrally placed genes of the network (Fig EV1C). MCOLN1 had the highest centrality score in the network and a concordantly high number of connections to other genes of the network (Fig 1H). These data suggest that MCOLN1 is a central node (i.e., a hub) in the network of upregulated endolysosomal genes in HRAS-driven cancers.

Is the expression of MCOLN1 dependent on the presence of oncogenic HRAS? To answer this question, we assessed MCOLN1 expression in T24 cells, which is a urinary tract cancer cell line with naturally occurring HRASG12V mutation [28]. In comparison with a bladder cancer cell line that is wild type for HRAS (HT1197 [29]), T24 cells exhibited ~3× increase in MCOLN1 expression (Fig 1I). Therefore, MCOLN1 expression in cancer cells is strongly correlated with the presence of oncogenic HRAS.

Cancer cells with oncogenic HRAS demonstrate elevated TRPML1 activity

To examine whether the increase in MCOLN1 expression has a functional consequence on TRPML1 channel activity, we performed Ca2+ imaging using fura-2 loaded HN31 cells. We evoked endolysosomal Ca2+ release by the sequential addition of the TRPML1 agonist, ML-SA1 [30], and the endolysosomolytic agent, glycyl-L-phenylalanine-2-naphthylamide (GPN) [31] in the absence of bath Ca2+ to ensure that entry from the extracellular medium did not contribute to the observed Ca2+ transients (Fig 2A and B). In comparison with cells stably expressing HRAS shRNA, peak amplitudes of ML-SA1-induced cytosolic Ca2+ transients were significantly larger in HN31 cells (Fig 2A–C and E). In contrast, peak amplitudes of GPN-induced transients—evoked after the TRPML1 transients had subsided—were significantly smaller in HN31 cells (Figs 2A, B, D and F). Consequently, ratios of amplitudes of the transients evoked by ML-SA1 and GPN were significantly higher in HN31 compared to cells stably expressing HRAS shRNA (Fig 2G). The increase in TRPML1-mediated endolysosomal Ca2+ release in HN31 cells was not a function of elevated endolysosomal Ca2+ content since the sums of the amplitudes of ML-SA1 and GPN transients—representative of the total endolysosomal [Ca2+]i—were not significantly different in HN31 and the HRAS shRNA-expressing variants (Fig 2H). Together, these data indicate that HRAS-dependent increase in MCOLN1 expression results in a proportional elevation of functional TRPML1 channels in HN31 cells (Fig 2I).

TRPML1 is required for the proliferation of cancer cells expressing oncogenic HRAS

Treatment of HN31 cells with siRNAs against MITF or TFEB led to significantly diminished cell proliferation (Fig EV2A) indicating a requirement for the two transcription factors in tumorigenesis. To examine the role of MCOLN1 expression, we generated HN31 cells that stably express a control shRNA or two independent shRNAs that decreased MCOLN1 expression (Fig EV2B). Compared to untreated cells or cells that expressed control shRNA, cells that expressed MCOLN1 shRNAs exhibited diminished proliferation (Fig 3A). Furthermore, MCOLN1 siRNA, which decreased the expression of the gene in HN31, T24, and HT1197 (Figs EV2B and C), diminished the proliferation of HN31 and T24 cells but not HT1197 cells (Fig 3A). The finding that proliferation of HT1197, which are wild type for HRAS, was insensitive to MCOLN1 knockdown is consistent with the notion that only the cancer cells with oncogenic HRAS are vulnerable to MCOLN1 knockdown. Vac14 functions with the enzyme PIKfyve to synthesize PI(3,5)P2 and, thereby, regulates TRPML1 activation [21,22]. Knockdown of VAC14 using two independent shRNA constructs, which led to the expected decrease in VAC14 mRNA (Fig EV2D), decreased the proliferation of HN31 cells (Fig 3B).

Figure 2. TRPML1-mediated endolysosomal Ca2+ release in cancer cells with oncogenic HRAS.
A, B Graphs showing the changes in the fura-2 ratio in cells of the indicated genotypes in response to ML-SA1 and GPN. Arrows serve as event markers that denote the points of time at which the drugs were added. Gray lines on top represent the time at which the bath Ca2+ was removed. All values represent mean ± SEM from 3 biological replicates.
C Same as the data shown in (A) except that the baseline ratios for both genotypes were adjusted to 0 at the start of the experiment. Lines on the right indicate peak amplitudes of Ca2+ release in response to the application of ML-SA1. All values represent mean ± SEM from three biological replicates.
D Same as (C) except that Ca2+ release was evoked using GPN.
E, F Peak amplitudes of Ca2+ release in cells of the indicated genotypes in response to ML-SA1 (E) and GPN (F). All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.
G Ratio of the amplitudes of Ca2+ release in cells of the indicated genotypes in response to ML-SA1 and GPN. All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.
H Total Ca2+ release in cells of the indicated genotypes in response to ML-SA1 and GPN. All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.
I Schematic representation of the notion that an increase in the expression of MCOLN1 results in a proportional increase in ML-SA1-induced Ca2+ release in HN31 cells.
Application of a pharmacological inhibitor of TRPML1 (ML-S1) [32] also significantly diminished proliferation of the HRAS<sup>G12V</sup> transformed oral cancer cells, UM-SCC-22A-HRAS<sup>G12V</sup> (Figs 3C and EV2E) [33]. In contrast, proliferation of UM-SCC-22A-HRAS<sup>WT</sup> cells, which express wild-type HRAS, was not affected by ML-S1 application (Figs 3C and EV2E). Furthermore, application of ML-S1 significantly attenuated the proliferation of HN31 and T24 cells but not HT1197 or the cells that stably express HRAS shRNA (Figs 3C and EV2E and F). The PIKfyve inhibitor, YM201636, which inhibits PI(3,5)P<sub>2</sub> synthesis and TRPML1 activation [34,35], also diminished...
Figure 3.
the proliferation of UM-SCC-22A-HRAS\textsuperscript{G12V} and HN31 cells (Fig EV2G). However, proliferation of cells that do not express oncogenic HRAS was not affected by YM201636 (Fig EV2G). These data indicate that proliferation of cancer cells expressing oncogenic, but not wild-type, HRAS is vulnerable to TRPML1 inhibition.

Next, we performed the chick chorioallantoic membrane (CAM) assay as an \textit{in vivo} xenograft model [36,37] to examine the effects of \textit{MCOLN1} knockdown and ML-SI1 application on the ability of HN31 cells to form tumors. CAM xenografts generated by growing human cancer cells histologically resemble tumors grown in mouse xenograft models [36,37]. These cost-effective, fast, and reproducible xenografts can be used to examine cancer cell proliferation using Ki67-labeling as an indicator and the influence of various drugs thereupon. In xenografts grown with HN31 cells expressing control shRNA, ~75% cancer cell nuclei were Ki67-positive (Fig 3D and E). In contrast, only ~30% of nuclei were Ki67-positive in xenografts grown with HN31 cells stably expressing an shRNA against MCOLN1 (Fig 3D and E). MCOLN1 knockdown also reduced the total number of cancer cells per field (Figs 3D and E). Similarly, application of ML-SI1 significantly decreased the fraction of Ki67-labeled nuclei (Appendix Fig S2A). However, the total number of HN31 cells per field was not significantly diminished in eggs treated with ML-SI1 (Appendix Fig S2B). Since ML-SI1 was added 1 and 3 days after the initial implantation of xenografts, our data suggest that subsequent attenuation of cell proliferation was not sufficient to significantly decrease cell numbers. On the other hand, implantation of HN31 stably expressing MCOLN1 shRNA likely proliferated slowly from the beginning, allowing for significant decreases in both Ki67-labeled cells and total cell number.

Expression of HRAS\textsuperscript{G12V}, but not HRAS\textsuperscript{WT}, was sufficient to potentiate MCOLN1 expression in UM-SCC-22A cells (Appendix Fig S2C). Furthermore, knockdown of MCOLN1 did not alter the proliferation of UM-SCC-22A-HRAS\textsuperscript{G12V} cells (Fig 3F). These data demonstrated reliable correlation between MCOLN1 expression and HRAS\textsuperscript{G12V}-driven cancer cell proliferation. Notably, ectopic overexpression of MCOLN1 in UM-SCC-22A-HRAS\textsuperscript{G12V} was sufficient to enhance the rate of cell proliferation (Fig 3F) indicating that MCOLN1 expression is necessary and sufficient for potentiation of cancer cell proliferation.

\textbf{MCOLN1 expression correlates with prognosis of BLCA and HNSC patients}

We asked whether the prognosis of cancer patients with tumors carrying activating mutations in HRAS correlated with MCOLN1 expression. In cases of BLCA and HNSC, stratifying patients with oncogenic HRAS mutations into high and low MCOLN1 expressors revealed that lower MCOLN1 expression correlated with significantly improved patient survival (Fig 3G). In patients that were wild type for HRAS, however, we found no correlation between MCOLN1 expression and patient prognosis (Fig 3G). Consistent with the idea that MTM1 encodes a lipid phosphatase that indirectly diminishes TRPML1 activity, higher expression of MTM1 correlated with improved patient survival (Appendix Fig S2D). Once again, in patients that were wild type for HRAS, we observed no correlation between MTM1 expression and patient prognosis (Appendix Fig S2D). Thus, patterns of gene expression predicted to favor elevated TRPML1 activity tracked with poorer survival of BLCA and HNSC patients with oncogenic HRAS mutations. The prognosis of THCA patients was generally very good with few fatalities being associated with this disease (Appendix Fig S2E). Accordingly, MCOLN1 expression was not related to survival in THCA patients either with or without HRAS mutations (Appendix Fig S2E).

\textbf{TRPML2 and TRPML3 exhibit partial roles in the proliferation of cancer cells with oncogenic HRAS}

TRPML2 and TRPML3 (encoded by MCOLN2 and MCOLN3, respectively) are TRPML1 paralogs, which heteromultimerize with TRPML1 and potentially influence its function [38]. Since ML-SI1 partially inhibits the TRPML1 paralogs [32], we assessed the contributions of TRPML2 and TRPML3 to HRAS-driven cancer cell proliferation. Neither MCOLN2 nor MCOLN3 were significantly altered in BLCA, THCA, and HNSC with mutant HRAS (Fig EV3A), although both genes were modestly upregulated in HN31 cells (Fig EV3B). Consistent with the changes in gene expression, knockdown of either MCOLN2 or MCOLN3 in HN31 cells led to slight (~10%) decreases in cell proliferation (Fig EV3C and D). Simultaneous knockdown of both MCOLN1 and MCOLN2, but not MCOLN1 and MCOLN3, additionally attenuated HN31 cell proliferation, i.e., MCOLN2 shRNA decreased the proliferation of cells with MCOLN1 knockdown by an additional ~10% (Fig EV3D). These data suggest non-redundant roles for TRPML1 and TRPML2 in cancer cell proliferation. Application of ML-SI1 after MCOLN1 knockdown decreased cell proliferation to the same extent as that induced by MCOLN2 shRNA (~10%), which is consistent with the “pan TRPML” effects of the drug [32].

\textbf{TRPML1 inhibition attenuated ERK phosphorylation in cells expressing oncogenic HRAS}

HRAS is a “molecular switch” that toggles between GTP or GDP-bound states [39]. In the GTP bound state, HRAS potentiates mitogenic pathways including the RAF–MEK–ERK kinase cascade.
Oncogenic mutations promote the active HRAS configuration and, thereby, evoke unrestricted ERK phosphorylation and cell proliferation. Indeed, stable overexpression of GFP-tagged HRASG12V in Madin-Darby Canine Kidney (MDCK) cells was sufficient to increase ERK phosphorylation (Fig 4A and B). ERK phosphorylation in HN31 cells was also dependent on oncogenic HRAS as evidenced by our finding that knockdown of HRAS decreased the pERK/ERK ratio (Fig 4C and E). Application of ML-S11 significantly reduced the pERK/ERK ratio, but only in those cells that expressed HRASG12V (Fig 4A and B). The pERK/ERK ratio was not changed in control MDCK cells in response to ML-S11. These effects of ML-S11 were not due to alterations in the expression levels of GFP-HRASG12V (Appendix Fig S3A–C). Knockdown of MCOLN1 in HN31 cells, but not cells stably expressing HRAS shRNA, also decreased ERK phosphorylation (Fig 4C and E, and Appendix Fig S3D and E). In T24 cells, either MCOLN1 knockdown or application of ML-S11 decreased ERK phosphorylation, whereas neither MCOLN1 knockdown nor ML-S11 influenced the pERK/ERK ratio in HT1197 cells, which are wild type for HRAS (Fig 4D and F). Taken together, these data demonstrate that pharmacological inhibition of TRPML1 or MCOLN1 knockdown attenuated ERK phosphorylation in only those cells that express oncogenic variants of HRAS.

**Effects of oncogenic RAS on cell proliferation and ERK phosphorylation is diminished in Drosophila lacking the MCOLN1 ortholog**

To further assess the relationship between RAS–ERK signaling and TRPML1 expression, we utilized Drosophila lacking the sole MCOLN1 ortholog, trpml [40], as a genetically tractable model. Loss of fly trpml leads to phenotypes that are remarkably similar to those observed in human cells lacking MCOLN1 [40]. Importantly, expression of human MCOLN1 suppressed mutant phenotypes when expressed in trpml null flies (trpml\(^{-}\)) [41]. We found that ectopic expression of the activated variant of the fly RAS homolog (dRasG12V) [42] in glia led to a significant increase in trpml expression (Fig 5A). Coexpression with GFP allowed us to detect dRasG12V-induced proliferation of glial cells in the larval brain (Fig 5B). Expression of dRasG12V and GFP in trpml\(^{-}\) glia led to a significantly attenuated phenotype (Figs 5B and C). Quantification of the relative volume of GFP-expressing cells in the larval brain indicated a \(~25\%\) decrease in glial volume in trpml\(^{-}\) expressing dRasG12V (Fig 5C).

Next, we asked whether dRasG12V-induced ERK phosphorylation is dependent on TRPML1. Because the fraction of glial cells in the larval brain is low (\(~10\%\) of the cell in the larval brain are glia), we examined ERK phosphorylation in larval macrophages and fat bodies, which constitute abundant sources of readily accessible cells. Expression of dRasG12V in larval macrophages led to increased levels of tubulin, total ERK, and ERK phosphorylation (Fig 5D and E and Appendix Fig S4), which is consistent with an increase in cell proliferation. The pERK/tubulin ratio (\(~8\times\) higher in dRasG12V, expressing macrophages) and pERK/ERK ratios were significantly attenuated in cells lacking trpml (Fig 5D and E, and Appendix Fig S4). The pERK/tubulin and pERK/ERK ratios were statistically indistinguishable in wild-type and trpml\(^{-}\) macrophages not expressing dRasG12V (Fig 5D and E, and Appendix Fig S4).

Since flies do not express an HRAS-equivalent gene, we ectopically expressed human HRASG12V in Drosophila larval fat bodies—an endocrine organ with features similar to human adipocytes and liver. HRASG12V led to \(~8\times\) increase in ERK phosphorylation (Fig 5F and G). These data demonstrate that activated human HRAS is able to couple with the appropriate signaling cascade in fly tissues and elicit ERK phosphorylation. As with dRasG12V, expression of human HRASG12V in trpml\(^{-}\) tissues led to dramatically diminished ERK phosphorylation (Fig 5F and G). Taken together, the data in flies agree with our findings in human cancer cells and demonstrate an evolutionarily conserved requirement for TRPML1 in RAS-induced ERK phosphorylation and cell proliferation.

**Inhibition of TRPML1 diminished clustering and localization of HRASG12V at the plasma membrane**

Activation of the RAF–MEK–ERK cascade depends on formation of RAS nanoclusters at the plasma membrane [43,44]. Accordingly, disruption of the clustering of oncogenic HRAS severely restricts downstream ERK phosphorylation and, consequently, limits cell proliferation. Given the effects of TRPML1 inhibition of ERK phosphorylation and cell proliferation, we used quantitative super-resolution electron microscopy (EM) spatial analysis to examine HRASG12V nanoclusters at the plasma membrane in response to ML-S11. We prepared intact basolateral plasma membrane sheets from GFP-HRASG12V expressing cells that were attached on EM grids with antibodies, which constitute abundant sources of readily accessible tissues led to dramatically diminished ERK phosphorylation (Fig 5D and E). Taken together, the data in flies agree with our findings in human cancer cells and demonstrate an evolutionarily conserved requirement for TRPML1 in RAS-induced ERK phosphorylation and cell proliferation.

![Figure 4. TRPML1 inhibition or MCOLN1 knockdown attenuated ERK phosphorylation in mammalian cells expressing activated HRAS.](image-url)
Figure 4.

**A** pERK/ERK in T24 cells (normalized to no siRNA) and tubulin.

**B** HRAS	extsuperscript{G12V} expressing controls (normalized to control DMSO).

**C** pERK/ERK in T24 cells (normalized to control DMSO) and tubulin.

**D** pERK/ERK in HT1197 cells (normalized to control siRNA) and tubulin.

**E** pERK/ERK in T24 cells (normalized to control siRNA) and tubulin.

**F** pERK/ERK in HT1197 cells (normalized to control DMSO) and tubulin.

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**Figure 4.**

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**Figure 5.**

**A**
- Control and dRasG12V relative *trpml* expression.

**B**
- Drosophila larval brain: Glial cell density increased in dRasG12V in trpml1.
- GFP in glial cells.
- VNC elongated.

**C**
- Bar chart showing relative glial volume normalized to wild-type.

**D**
- Western blot analysis of pERK and ERK in control and dRasG12V in macrophages.

**E**
- Bar chart showing relative pERK/tubulin normalized to wild-type control.

**F**
- Western blot analysis of pERK in control and HRASG12V in macrophages.

**G**
- Western blot analysis of pERK, ERK, and tubulin in control and HRASG12V in macrophages.
1 μm²—an estimate of the level of GFP-HRAS<sup>G12V</sup> on the cell surface—was significantly reduced in ML-SI1-treated cells (Fig 6C).

Lateral segregation of GDP- and GDP-bound HRAS depends on the levels of plasma membrane cholesterol [45,46]. Interestingly, nanoclustering of HRAS<sup>G12V</sup> was significantly restored in ML-SI1-treated cells upon addition of exogenous cholesterol (Fig 6B). In contrast, addition of phosphatidylserine (PS), a major lipid constituent of the inner leaflet of the plasma membrane that is required for KRAS<sup>G12V</sup> clustering [47], did not restore the clustering of HRAS<sup>G12V</sup> (Fig 6B). Addition of exogenous cholesterol also restored the plasma membrane localization of HRAS<sup>G12V</sup> as evidenced by the number of gold particles in the plasma membrane sheets (Fig 6C). Knockdown of MCOLN1 also decreased both the clustering and plasma membrane levels of GFP-HRAS<sup>G12V</sup> (Figs 6D and E, and EV4A). Furthermore, exogenously added cholesterol was sufficient to elevate the clustering of GFP-HRAS<sup>G12V</sup> in control cells and prevented MCOLN1 knockdown from attenuating GFP-HRAS<sup>G12V</sup> clustering (Fig 6D). Application of a statin (20 μM Simvastatin) also diminished GFP-HRAS<sup>G12V</sup> clustering and levels at the plasma membrane (Fig 6D and E), which demonstrates necessity for cholesterol in HRAS<sup>G12V</sup> clustering. Lastly, exogenous application of cholesterol for 3 h was sufficient to ablate the differences in ERK phosphorylation observed in HN31 cells that express MCOLN1 shRNA (Fig 6F and G). The cholesterol-induced increase in ERK phosphorylation in control HN31 cells (Fig 6F and G) likely reflects the increase in HRAS<sup>G12V</sup> clustering evoked by exogenous cholesterol (Fig 6D).

ML-SI1 and statin also decreased the nanoclustering of GFP-HRAS<sup>WT</sup> (Fig EV4B). Diminished GFP-HRAS<sup>WT</sup> clustering in ML-SI1-treated cells was fully restored by exogenous cholesterol (Fig EV4B). Intriguingly, neither ML-SI1 nor statin influenced the levels of GFP-HRAS<sup>WT</sup> in the inner leaflet of the plasma membrane (Fig EV4C). These data indicate that both the nanoclustering and optimal plasma membrane localization of HRAS<sup>G12V</sup> depend on TRPML1 (Fig 6H), whereas only the clustering of wild-type HRAS was sensitive to lowered TRPML1 function (Fig 6H). Given that both plasma membrane localization and nanoclustering affect the signal output of HRAS [48–50], the distinct effects of TRPML1 inhibition on HRAS<sup>G12V</sup> compared to HRAS<sup>WT</sup> explain the selective vulnerability of oncogenic HRAS-expressing cancer cells to TRPML1 inhibition.

To validate the necessity for cholesterol in HRAS<sup>G12V</sup>-dependent cell proliferation, we assessed the effects of cholesterol depletion on HN31 cell proliferation. Consistent with critical roles for membrane cholesterol in myriad cellular signaling events, proliferation of both control HN31 cells and HN31 cells expressing HRAS shRNA was diminished by β-cyclodextrin albeit with differing sensitivities (Fig EV5A). Analyses of the dose sensitivities of β-cyclodextrin-induced decrease in cell proliferation revealed that HN31 cells expressing HRAS shRNA exhibited significantly higher repression coefficient (K<sub>i</sub>) values than control HN31 cells (Fig EV5B). Similarly, proliferation of HN31 cells and the variants stably expressing HRAS shRNA were diminished when the cells were grown in the presence of different doses of statin (Fig EV5C), with HN31 cells expressing HRAS shRNA, ∼2× higher K<sub>i</sub> values (Fig EV5D). These data indicate that cancer cells with oncogenic HRAS exhibited greater sensitivity to cholesterol depletion and thus point to a necessary role for cholesterol in the proliferation of those cells.

**TRPML1 is required for processing and trafficking of cholesterol in endolysosomes**

Cholesterol trafficking is intimately coupled to endolysosomal function. Lysosomal proteins such as NPC1 and NPC2 have well-established roles in cholesterol extrusion from endolysosomes [51]. Since TRPML1 is required for the exocytosis of endosomal vesicles (Fig 7A) [32,52,53], it stands to reason that TRPML1 mediates the recycling of endolysosomal cholesterol back to the plasma membrane (Fig 7A). Additionally, TRPML channels are required for the fusion of late endosomes with lysosomes [54], which is necessary for de-esterification of endocytosed cholesterol esters (Fig 7A). Thus, we hypothesized that inhibition of TRPML1 would result in diminished plasma membrane cholesterol levels stemming from aberrant intracellular accumulation of cholesterol and inadequate de-esterification of cholesterol esters.

Although neither ML-SI1 nor shRNA-mediated MCOLN1 knockdown affected the levels of total cholesterol in HN31 cells (comprised of both free and esterified cholesterol) (Fig 7B), the fraction of free cholesterol was significantly decreased in response to either perturbation (Fig 7C). A decrease in free cholesterol without significant changes in total cholesterol is consistent with diminished de-esterification. Similarly, the fraction of free cholesterol was ∼25% lower in *Drosophila* brain tissues lacking *trapml* (Fig 7D). Thus, TRPML proteins have an evolutionarily conserved role in cholesterol de-esterification and maintenance of free cholesterol levels.
Figure 6.
To assess subcellular distribution of cholesterol, we examined the localization of a fluorescently tagged sensor of cholesterol, mCherry-D4H [55]. As described previously [8], confocal imaging of MDCK cells revealed that the majority of mCherry-D4H decorated the plasma membrane of the cells (Fig 7E). However, within 24 h of ML-SI1 application, most of the mCherry-D4H signal relocated to intracellular vesicles leading to a significant increase in the number of intracellular mCherry-D4H puncta (Figs 7E and F). We found that the mCherry-D4H puncta colocalized with endolysosomal membrane protein, LAMP1-GFP (Fig 7G), which indicates that TRPML1 inhibition caused the cholesterol sensor to be trapped in endolysosomes. In HN31 cells, we evaluated the distribution of cholesterol using the cholesterol-binding polyene antibiotic, filipin [56], as a fluorescent. We found that MCOLN1 knockdown led to redistribution of filipin from the plasma membrane to intracellular punctae (Fig 7H), which we quantified as an increase in the fraction of cellular area stained with filipin (Fig 7I). ML-SI1 treatment also led to the mislocalization of the recombinant sphingomyelin sensor, GFP-lysensin [57], from the plasma membrane to internal vesicles (Fig EV5E). Since sphingomyelin resides in cholesterol-enriched microdomains, these data demonstrate that inhibition of TRPML1 leads to the anomalous accumulation of cholesterol-laden microdomains in endolysosomes. Taken together, these data demonstrate that inhibition of TRPML1 leads to a redistribution of cholesterol.

Concomitant with aberrant endolysosomal localization of mCherry-D4H and GFP-lysensin, ML-SI1 treatment depleted these markers from the plasma membrane (Figs 7E and EV5E). To examine whether biophysical properties of the membrane in ML-SI1-treated cells reflect the depletion of cholesterol from the plasma membrane, we prepared giant plasma membrane vesicles (GPMVs) [58] from control and ML-SI1-treated cells. Lipid constituents of GPMVs closely mimic those of the plasma membrane, and phase separation of raft and non-raft markers in GPMVs reflects the propensity for cholesterol-induced ordered domain formation in mammalian membranes [59]. We found that GPMVs isolated from ML-SI1-treated cells exhibited a decrease in the phase separation temperature (miscibility temperature) in comparison with GPMVs isolated from vehicle-treated cells (Figs 7J and K). Since miscibility temperature is related to the lifetime of membrane nanodomains and is regulated by cholesterol content [60], our data indicate that TRPML1 inhibition significantly influences the physical properties of the plasma membrane via disruption of cholesterol content of the plasma membrane. Consistent with this deduction, direct cholesterol depletion has a similar effect of reducing the fraction of phase-separated GPMVs [60]. Further supporting a role for cholesterol, we found that exogenous application of cholesterol prevented the decrease in GPMV miscibility temperature in ML-SI1-treated cells, whereas exogenous cholesterol did not influence the miscibility temperature of GPMVs generated from vehicle-treated cells (Fig 7J and K). These data indicate that ML-SI1 treatment results in alteration of plasma membrane physical properties in a manner consistent with diminished cholesterol levels in that compartment.

Discussion

Using a combination of bioinformatic analyses of human tumors and experimental assessment of multiple cancer cell lines, we identified a gene expression signature in HRAS-positive tumors that points to involvement of the Mitf/Tfe3/Tfeb transcription factors [14-16,61]. Indeed, expression of MITF, TFEB, and TFE3 was elevated in cells with oncogenic HRAS mutations. Concordantly, knockdown of either MITF or TFE3 led to a decrease in the proliferation of the HN31 oral cancer cell line. These findings, which suggest partially redundant functions of these transcription factors in cancer cell proliferation, are in agreement with previous reports of Mitf/Tfe3/Tfeb-induced endolysosomal biogenesis in potentiation of tumorigenesis [9,62].

How could we leverage this understanding to develop strategies to mitigate the activation of these transcription factors in cancer? Since drugs that directly block Mitf/Tfe3/Tfeb are not available, indirect approaches via stimulation of mTORC1 or inhibition of calcineurin to arrest nuclear import of these transcription factors [15,26,63] could mitigate unremitting endolysosomal biogenesis. However, cancer-related alterations preemptively decouple the nucleocytoplasmic transport of Mitf/Tfe3/Tfeb from mTORC1 [9], potentially
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Figure 7.
making the mTORC1–transcription factor axis less tractable. As an alternative, one could specifically ablate the downstream genes that encode agents of disease. In the case of pH-responsive endolysosomal proteins, dissipation of vesicular acidity may mimic gene ablation. The latter rationale has guided chemotherapeutic strategies that espouse endolysosomal alkalization using chloroquine [12]. However, since many endolysosomal proteins are pH sensitive, neutralizing vesicular acidity could elicit unintended outcomes.

Targeting endolysosomal genes that are selectively upregulated in a particular type of cancer might be an appropriately nuanced strategy to suppress tumorigenesis. Since principal endolysosomal culprits could vary between cancers, a priori knowledge of gene expression profiles in different cancers would be needed. To test this idea, we analyzed patterns of endolysosomal gene expression using oncogenic HRAS-driven BLCA, HNSC, and THCA as a model. Network analyses of overexpressed endolysosomal genes, many of which are under the control of Mitf/Tie3/Tf3b, pointed to an important role for MCOLN1. Expression of oncogenic HRAS was both necessary and sufficient for eliciting the MCOLN1 overexpression in the human cancer cell lines we examined. Consistent with increased MCOLN1 expression, TRPML1-mediated endolysosomal Ca\(^{2+}\) release was elevated in cancer cells with oncogenic HRAS mutations. Interestingly, expression of the activated variant of Drosophila Ras also led to increased expression of the MCOLN1 ortholog, trpml, indicating an evolutionarily conserved role for RAS–ERK signaling in the expression of TRPML1s. Furthermore, MCOLN1 knockdown diminished the proliferation of cancer cells that expressed oncogenic HRAS. Pharmacological inhibition of TRPML1—directly with ML-SI1 or indirectly by blocking PI(3,5)P\(_2\) synthesis—also restricted the proliferation of human cancer cells carrying oncogenic HRAS mutations. Increased cell proliferation and RAS–ERK signaling observed in Drosophila cells expressing activated variants of dRas or human HRAS were significantly attenuated in animals lacking the TRPML1 ortholog. Interestingly, ectopic overexpression of MCOLN1 in oral cancer cells that were wild type for HRAS led to an increase in cell proliferation, which raises the intriguing notion that oncogenic HRAS drives cancer cell proliferation in part by upregulating MCOLN1 expression.

Neither MCOLN1 knockdown nor TRPML1 inhibition affected the proliferation of cells without oncogenic HRAS. We demonstrated the selective vulnerability of HRAS-driven cancer cells to decreased TRPML1 function in bioinformatic analyses of patient prognosis, two separate oral cancer cell lines, and a bladder cancer cell line. Stratification of HNSC and BLCA patients with oncogenic HRAS mutations on the basis of MCOLN1 or MTM1 expression revealed that higher MCOLN1 or lower MTM1 expression correlated with poorer patient prognosis. The correlations between MCOLN1 and MTM1 expression and patient survival were not observed in HNSC or THCA patients that were wild type for HRAS.

From a mechanistic standpoint, we found that inhibition of TRPML1 diminished the formation of HRAS\(^{G12V}\) nanoclusters and the levels of the protein in the plasma membrane. Whereas nanoclustering of the wild-type protein was also diminished upon TRPML1 inhibition, we did not detect a decrease in the plasma membrane levels of HRAS\(^{WT}\) by ML-SI1. Given that both plasma membrane localization and nanoclustering affect the signal output of HRAS [48–50], our data suggest that the selective loss of HRAS\(^{G12V}\) from the plasma membrane underlies the specificity of TRPML1 (please refer to model in Fig 6H). The effects of MCOLN1 knockdown or TRPML1 inhibition on HRAS\(^{G12V}\) clustering and localization could be restored by the ectopic addition of cholesterol. Indeed, inhibition of TRPML1 decreased cellular cholesterol levels, especially at the plasma membrane, due to the combined inhibition of cholesterol de-esterification and recycling of cholesterol-laden endolysosomal vesicles back to the membrane. The necessity for cholesterol in the proliferation was further demonstrated by application of TRPML1-independent cholesterol depletion agents.
This study adds to the growing body of knowledge regarding endolysosomal proteins in oncogenesis. A recent study reported that increased expression of a TRPML1 paralog, TRPML2, supports tumorigenesis in glioma [64]. We also found that the gene encoding TRPML2 is slightly elevated in HN31 cells and plays a minor role in cancer cell proliferation. Other endolysosomal transporters and channels have also been implicated in cancer progression [65]. Our findings are also reminiscent of the reports that inhibition of lysosomal acid sphingomyelinase attenuates ERK signaling and tumorigenesis [8,10,11]. The overlap in the phenotypes associated with inhibition of acid sphingomyelinase and TRPML1 raises the question of whether these proteins experience functional interdependence. Since activity of TRPML1 is subject to regulation by a diverse group of membrane lipids [30], it is plausible that sphingomyelins influence TRPML1 function. From a therapeutic perspective, it would be worthwhile to assess whether simultaneous inhibition of TRPML1 and acid sphingomyelinase could synergistically deter tumorigenesis. It is also possible that a combinatorial approach of simultaneous inhibition of both proteins could permit usage of subthreshold drug concentrations and thereby minimize the potential side effects associated with the administration of either drug alone. In summary, we anticipate that insights gleaned from this study could be leveraged to design novel anti-cancer therapeutic strategies.

Materials and Methods

Bioinformatic and survival analyses

We examined the expression of 210 endolysosomal genes in BLCA, HNSC, and THCA tumors using TCGA datasets available at the UCSC Xena functional genomics browser (https://xenabrowser.net). We obtained mRNA seq counts for each gene in tumors bearing oncogenic HRAS mutations (missense mutations at codons 12, 13, 61, and 117) and matched normal tissues. We calculated the Z-scores for each gene in every tumor using the formula:

\[
Z(gene, tumor) = \frac{(mRNA \text{ seq counts in tumor}) - (average \text{ of mRNA seq counts in normal tissue})}{\text{standard deviation of mRNA seq counts in normal tissue}}
\]

After calculating individual Z-scores by comparing tumors and matched normal tissues, we combined the Z-values for further analyses.

To evaluate correlated patterns of gene expression in tumor samples, we generated a gene-by-gene matrix comprised of Pearson's coefficients of correlation between samples, we generated a gene-by-gene matrix comprised of Pearson's coefficients of correlation between samples. To analyze network topology, we used CytoScape. For these analyses, gene names were network nodes and Pearson's correlations of gene expression represented the edge values. To analyze the network, we treated it as undirected. The network shown in Fig 1G was generated using the “Attribute Circle Layout” on the basis of closeness centrality. The closeness centrality scores were used to rank the endolysosomal genes to generate the GSEA dataset. The nominal P and FDR q values were obtained after running the “GSEA Perranked” algorithm using the following parameters: (i) number of permutations = 1,000; (ii) enrichment statistic = weighted_p2; and (iii) normalization mode = meanadj.

For survival analyses, we separated the high and low expressors for each gene into the top and bottom 50% on the basis of Z-scores. Subsequently, we used the log-rank (Mantel-Cox) test to determine the P-values for each gene.

Cell culture

All cell lines were maintained at 37°C under 5% CO2. We cultured MDCK and UMSCC-22A cells in DMEM containing GlutaMAX (Gibco) that was supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen), and penicillin-streptomycin. We cultured HN31, HT1197 (obtained from ATCC), and T24 (obtained from ATCC) cells in DMEM high glucose media (Fisher Scientific) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen), penicillin-streptomycin, non-essential amino acids, pyruvate, and vitamins (all from Fisher Scientific). For the experiments involving drug treatment, we added DMSO (vehicle) or the drugs directly to the culture media.

Endolysosomal gene expression analyses

To evaluate the expression of endolysosomal genes, we used the primer sets indicated below. For performing RT-qPCR, we first extracted RNA using the RNeasy Mini Kit (Qiagen) as per the manufacturer’s protocol. After quantification of RNA, we generated cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer’s protocol. We performed RT-qPCR using SYBR Green JumpStart Taq ReadyMix (Sigma) as per the manufacturer’s protocol.

CTSA:
Forward: 5'-CAGGCTTTGCTCTCTCTCAGA-3'
Reverse: 5'-TCACGATTCAGGCTCTTTTGA-3'
CTSD:
Forward: 5'-ACGTGGACATCGCTTGCT-3'
Reverse: 5'-CATTCTTCGGATGGCAGTGA-3'
GAPDH:
Forward: 5'-GAAGGTGAAGGTCGGAGTC-3'
Reverse: 5'-GAAGATGTCCTGATGGGATTTC-3'
GBA:
Forward: 5'-CAGGCTTTGCTCTCTCTCAGA-3'
Reverse: 5'-CCCGTGGTATTAGCCTGGAT-3'
HEX:
Forward: 5'-ACGGCAAAACAATCTTCTTCAG-3'
Reverse: 5'-CCGTATCGTGACCTGTTTT-3'
LAMPI:
Forward: 5'-AGTTACACCGCTCCAGCTCAT-3'
Reverse: 5'-TTCTTTGAGCTCGCATTTG-3'
MCOLN1:
Forward: 5'-CTGGTGCTACCGGTGAG-3'
Reverse: 5'-CTGCTCCGGGCTTAGG-3'
Ca²⁺ imaging

Cells were trypsinized and seeded onto glass-bottom dishes (In vitro Science). The following day, cells were loaded with 10 μM fura-2-AM (Invitrogen) in culture medium for 1 h. Subsequently, the medium with fura-2-AM was replaced with 150 μL of bath solution (125 mM NaCl, 5 mM KCl, 10 mM MgSO₄, 10 mM KH₂PO₄, 1 mM CaCl₂, 5.5 mM glucose, and 5 mM HEPES in pH 7.4). Next, dishes containing the cells were mounted on the stage of a wide-field fluorescence imaging system (Nikon). After baseline fluorescent responses were acquired for ~1.5 min, 50 μL of bath solution was pipetted out of the dishes to dilute MLSA-1 (Tocris) and GPN (Cayman) and added back to the bath in dishes. Fura-2 signals were recorded by 340/380 nm excitation and 510 nm emission using NIS Elements imaging software (Nikon).

Analyses of cell proliferation

Cell proliferation was determined using two independent assays: CyQuan proliferation assay (Thermofisher) and WST-1 (4-(3-(4-i‐

MCOLN2:
Forward: 5′-CGGCAGCCTTATCGTTTCC-3′
Reverse: 5′-GCCATTGCTATCTGACGGT-3′

MCOLN3:
Forward: 5′-TCTCTCCCTGCTGACTCTG-3′
Reverse: 5′-CAGGATCTGCCATCTCTGGG-3′

MITF:
Forward: 5′-GAAATCTGGGCTGATTGGA-3′
Reverse: 5′-AGGAGTTGCTGATGTCAGG-3′

TFEB:
Forward: 5′-CCAGAAGCCAGACCTCAGAT-3′
Reverse: 5′-TTGTATTGCTTCTTCTTCCGG-3′

TFE3:
Forward: 5′-AGGATCAAGACGCTGGGAC-3′
Reverse: 5′-CCGGCTCCTCCAGCTACAC-3′

Drosophila trpml:
Forward: 5′-ACGCGAATGTAAGCCGATCT-3′
Reverse: 5′-ACGCGAATGTAAGCCGATCT-3′

Drosophila actSC:
Forward: 5′-TGTGATTGTCTTTCTTCTGCCG-3′
Reverse: 5′-TGTGATTGTCTTTCTTCTGCCG-3′

growth medium. Cells were cultured in the indicated concentrations of statins. To determine the Kᵢ values, we used the Hill input function for a repressor:

\[
f(X) = \frac{\beta}{1 + \left(\frac{X}{X_0}\right)^n}
\]

This equation was modified to:

\[
\frac{1}{f(X)} = \frac{1}{\beta} + \frac{1}{\beta K_i X^n}
\]

The parameter \(f(X)\) was the relative change in cell numbers normalized to the sample with no drug (%), \(\beta\) was the maximal value of \(f(X)\) (100%), \(X\) was the drug concentration, and \(n\) was the Hill coefficient, which was determined empirically by comparing linear fit of the curves generated by plotting 1/f(X) against \(X^n\). For \(\beta\)-cyclodextrin, Hill coefficient = 4 (Fig EV5B). For statins, we used the Hill coefficient values of 2 and 5 for HN31 cells and HN31 variants stably expressing HRAS shRNA, respectively.

CAM xenografts

Fertilized and specific pathogen-free chicken eggs were purchased from Charles River (Norwich, Connecticut). The chick chorioallantoic membrane (CAM) was accessed and prepared as described previously [37]. On embryonic day 7, the eggs were inoculated with 10⁶ HN31 cells per egg. Three groups of eggs were topically treated on days 1 and 3 after inoculation with 100 μl of DMSO or ML-SI1 at the concentrations of 10 μM or 30 μM. On the 4th or 7th day after inoculation, eggs were humanely euthanized via hypothermia as per American Veterinary Medical Association (AVMA) guidelines (https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx). The tumors were excised and fixed in 10% formalin followed by paraffin embedding. Slides made from these blocks were stained with hematoxylin and eosin, and anti-Ki67 antibody (MIB-1, Biocare, Pacheco, CA, USA). The HN31 cell nuclei in the sections can be easily distinguished from the host cell nuclei due to the significantly larger size of the former. For quantification, we counted the numbers of Ki67-positive and Ki67-negative cells in 8–10 fields (using a 10× objective) per slide. These numbers allowed us to calculate the fraction of Ki67 nuclei as well as the total number of nuclei per field. Each slide was from a different egg, and the number of these is indicated as data points in the bar graphs.

Gene knockdown by RNA interference

The siRNA sequences used for targeted silencing of human MCOLN1, MITF, and TFEB were designed as described previously [67–69] and custom synthesized as ON-TARGET plus constructs by Sigma-Aldrich. The following sequences were used:

MCOLN1—5′-CCCCACATCCCCAGGTAGTAA-3′
MITF—5′-AGCAGUACCUUUCUACCACTT-3′
TFEB—5′-CCGCCTGGAGATGACCAACAA-3′

Control siRNA (SC001, Sigma-Aldrich) was used as a negative control. Six-well plates were transfected using X-tremeGENE 9 DNA transfection reagent (Roche Applied Science). The following day, cells were loaded with 10 μM fura-2-AM (Invitrogen) in culture medium for 1 h. Subsequently, the medium with fura-2-AM was replaced with 150 μL of bath solution (125 mM NaCl, 5 mM KCl, 10 mM MgSO₄, 10 mM KH₂PO₄, 1 mM CaCl₂, 5.5 mM glucose, and 5 mM HEPES in pH 7.4). Next, dishes containing the cells were mounted on the stage of a wide-field fluorescence imaging system (Nikon). After baseline fluorescent responses were acquired for ~1.5 min, 50 μL of bath solution was pipetted out of the dishes to dilute MLSA-1 (Tocris) and GPN (Cayman) and added back to the bath in dishes. Fura-2 signals were recorded by 340/380 nm excitation and 510 nm emission using NIS Elements imaging software (Nikon).
Transfection Reagent (Roche). Transfections were performed as described by the manufacturer’s protocol. All knockdowns were confirmed by RT-qPCR. The shRNA sequences used were as follows:

**MCOLN1:**
Target sequence 1: 5’-GGCTGTTTCTGCTCATACATTG-3’
Target sequence 2: 5’-CTCGTGTATCAGTGTGACATGACACT-3’

**MCOLN3:**
5’-CCGGCCATGCCAGTTTTTG-3’
5’-GGCTATGCTTTTTTG-3’

**GATCTGGATTTTTG-3’**
CATGCCAGTTTTTG-3’

**5’-GCATGCCAGTTTTTG-3’**
CATGCCAGTTTTTG-3’

**VAC14:**
5’-CCGGTCCAGATCCTGGGTGACAATGCTCGAGCATTGTCACCCAGGATCTGAGATT TG-3’

The control shRNA was SHC002—non-target shRNA.

We produced lentiviral particles with a 3rd generation system and used HEKT 293T cell for packaging. Briefly, one day before transfection, we seeded HEKT 293T cells in full growth media into 6-well plates at the appropriate density to achieve ~60% confluency at the time of transfection. We added 10 μg of total DNA per transfection (shRNA-containing vector = 1.6 μg, pMDLg/pPRE = 4 μg, pRSV-Rev = 2 μg, pMD2.g + 2.4 μg) and used a 4:1 ratio of XtremeGENE 9 DNA Transfection Reagent (Roche). We incubated the cells to incubate (37°C, 5% CO2) the cells for 18 h before adding media with high serum. We subsequently removed the methamine bromide (Sigma) for 6 h. We subsequently removed the treatment media and replaced it with full growth media. We allowed the cells to incubate (37°C, 5% CO2) for at least 24 h before adding puromycin-containing media (2 μg/ml) for selection of cells expressing shRNA.

**Western blotting**

When generating cellular extracts for Western blotting, we harvested cells in a lysis buffer consisting of 10% NP40, 1 M Tris (pH 7.5), 5 M NaCl, 0.5 M MgCl2, 0.5 M EGTA (pH 8.0), 5% NaF, Aprotinin (1 mg/ml), 50 mM Na3VO4, Leupeptin (1 mg/ml), and 1 M DTT. We loaded cell or Drosophila extracts onto 4–20% gradient Tri-glycine gels (Bio-Rad) for SDS-PAGE. After transfer to nitrocellulose membranes, the blots were blocked using the Odyssey blocking buffer (LI-COR Biosciences) followed by incubation with primary antibodies and secondary antibodies described below. All blots were imaged using the Odyssey imaging platform (LI-COR Biosciences). For quantification, we determined band intensities using ImageJ (NIH). When probing the blots with antibodies from the same species, we stripped the blots using the NewBlot™ nitrocellulose stripping buffer as per the manufacturer’s instructions (LI-COR Biosciences). All antibody incubations were performed in the Odyssey blocking buffer (LI-COR Biosciences).

Primary antibodies used were rabbit anti-TFEB (Bethyl laboratories) [26], rabbit anti-pERK (Cell Signaling), rabbit anti-ERK (Cell Signaling), and mouse anti-β-tubulin (E7, DSHB). Secondary antibodies were used 680LT anti-rabbit and IRDye 800CW anti-mouse (LI-COR Biosciences).

**Drosophila husbandry and stock generation**

We reared Drosophila on standard fly food (1 l of food contained: 95 g agar, 275 g Brewer’s yeast, 520 g of cornmeal, 110 g of sugar, 45 g of propionic acid, and 36 g of Tegosept) as described previously [70]. The fly lines used in these studies were Oregon R, repo-GALA [71], UAS-med8GFP [72], UAS-dRasG12V and he-GAL4 [73], cg-GALA [74].

To generate the UAS-HRasG12V flies, the DNA sequence encoding GFP- and HA-tagged human HRasG12V was PCR-amplified using the primers: forward—ATGGTGAGCAAGGGCGAGGAG; reverse—TTAGGAGACACACTTGCCAGC. Amplified fragments were subcloned into pUAS-attB vector using the In-fusion cloning kit (Clontech). The resulting construct, pUAS-GFP-HA-HRasG12V, was inserted into a Drosophila 2nd chromosomal docking site carrying PBac[y+-attP-3B]VK00001.

**Experiments using Drosophila**

We established crosses with adults of the appropriate genotypes in vials placed in an incubator maintained at 25°C. After clearing the parental adults within a week of setting up crosses, we isolated wandering 3rd-instar larvae from the vials for further analyses. For the experiments involving determination of glial cell volumes, we filleted and fixed the larvae expressing membrane bound GFP and/or dRasG12V in control or trpml1 larvae using PBS + 4% paraformaldehyde for 30 min. After washing the fillets in PBS (3×), we placed the samples in VECTASHIELD mounting media containing DAPI (Vector labs). We imaged the samples using a Nikon A1 confocal microscope using a 20× air objective, with the Z plane thickness set to 1 μm. Subsequently, we generated 3D reconstructions of the larval brains expressing GFP in glia and determined the volume of these reconstructions using Imaged (NIH).

For analyzing larval macrophages, we tore the body walls of 3rd-instar larvae in depression slides containing 2× Laemmli buffer to simultaneously release and solubilize the cells. We dissected larval fat bodies in PBS in depression slides, after which we removed the PBS and added 2× Laemmli buffer to solubilize the samples.

**EM-univariate spatial analysis**

The Ripley’s univariate K-function analysis tests the null hypothesis that all points with a selected 2D area are distributed randomly [43,47]. We seeded MDCK cells stably expressing GFP-HRasG12V on pioform- and poly-L-lysine-coated gold EM grids to a confluent monolayer. Next, we fixed intact basolateral plasma membrane sheets from the MDCK cells attached to the EM grids with 4% paraformaldehyde (PFA) and 0.1% glutaraldehyde, followed by immunolabeling with 4.5 nm gold nanoparticles conjugated to anti-GFP antibody. Finally, we negative-stained the grids with uranyl acetate and embedded in methyl cellulose. Gold nanoparticles on the intact basolamella membrane sheets were imaged using TEM at...
containing the samples mixed with the reaction mixture was incubated at 37°C for 60 min. Fluorescence signals (excitation/emission = 535/590 nm) were measured by an Infinity M200 plate reader (Tecan). We determined the free cholesterol in each sample using a standard curve.

Transfection and imaging of cultured cells

We seeded MDCK cells stably expressing mCherry-D4H on glass coverslips 15–20 h before transfection with plasmids carrying LAMP1-mGFP. We transfected the cells in serum-free culture medium using X-tremeGENE 9 transfection reagent (Sigma-Aldrich) according to the manufacturer’s instructions. 24 h after transfection, we replaced the culture medium with growth medium containing 10% fetal bovine serum.

After drug treatment, we fixed the cells on coverslips using PBS + 4% paraformaldehyde for 30 min. After washing off the fixative, we mounted the coverslips in VECTASHIELD mounting media containing DAPI (Vector labs). We imaged the coverslips using a Nikon A1 confocal microscope using a 40× oil objective. We counted the number of mCherry-D4H puncta in Z-projections of confocal images using ImageJ (NIH).

To visualize sphingomyelin, we applied a recombinant fragment of lysenin protein tagged with GFP as described [55]. Briefly, live MDCK cells were incubated with 60 μg/ml MBP-GFP-lysenin (amino acids 161-297 of full-length lysenin) for 15 min at room temperature in serum-free medium. Subsequently, we fixed cells with 4% paraformaldehyde (PFA). In another set of experiments, we first fixed and permeabilized the cells using 0.05% saponin before incubation with 60 μg/ml MBP-GFP-lysenin and DAPI for 15 min at room temperature. All samples were then imaged in a Nikon A1 confocal microscope.

Subconfluent (60–70%) cells grown on glass bottom dishes (In Vitro Science) were incubated with DMEM supplemented with 10% FBS. One day after seeding, cells treated with DMSO or 10 μM ML-S11. After 24 h incubation, filipin staining of formaldehyde-fixed cells was performed to assess the effect of manipulations on membrane cholesterol. In brief, cells were fixed in 4% formaldehyde in PBS for 15 min followed by incubation in PBS containing 125 μg/ml filipin (Sigma-Aldrich) for 30 min at room temperature. All samples were then imaged in a Nikon A1 confocal microscope.

Isolation and analysis of GPMVs

We isolated GPMVs and calculated the miscibility temperature as described [58,59,77]. Briefly, cells were washed with PBS and stained with 5 μg/ml FAST-DiO (Invitrogen), a green fluorescent lipid dye that strongly partitions to the disordered phase, for 10 min on ice. Then, the cells were washed twice with GPMV buffer (10 mM HEPES, 150 mM NaCl, 2 mM CaCl2, pH 7.4) and incubated with 25 mM PFA and 2 mM DTT for 1 h at 37°C to induce GPMV formation. The GPMV-rich suspension was then decanted and placed in a temperature-regulated imaging chamber. GPMVs were imaged from 4°C to 28°C, counting phase-separated and uniform vesicles at each temperature. For each temperature, 25–50 vesicles were counted and the percent of phase-separated vesicles were calculated, plotted versus temperature, and fitted to a sigmoidal curve to determine the temperature at which 50% of the vesicles possessed a value of 1 when $K(r) = An^{-2} \sum_{i,j} w_{ij}(||x_i - x_j|| \leq r)$

$L(r)/C_0^n = \frac{L(r) - r}{\sqrt{k/C_20}}$

where $K(r)$ is the univariate spatial distribution analysis for n gold particles within an intact plasma membrane area of A; r is the length scale between 1 and 240 nm; || || is the Euclidean distance, which possesses a value of 1 when $||x_i - x_j|| \leq r$ or a value of 0 when $||x_i - x_j|| > r$. The parameter $w_{ij}^{-1}$, which is used for an unbiased edge correction, defines the fraction of the circumference of a circle with the center at $x_i$ and radius $||x_i - x_j||$. Monte Carlo simulations estimate the 99% confidence interval (99% CI) of $K(r)$, which is used to normalize $L(r) - r$ linearly transformed from $K(r)$. A $L(r) - r$ value of 0 indicates gold distribution is completely random. A $L(r) - r$ value above the 99% CI of 1 indicates statistical clustering.

For each condition, at least 15 plasma membrane sheets were imaged and analyzed. We use bootstrap tests to compare our calculated point patterns to 1,000 bootstrap samples to evaluate the statistical significance between drug-treated and drug-untreated groups as described [75].
were phase-separated (i.e., miscibility temperature), which is demonstrated by the dashed vertical lines in Fig 7J.

Statistical methods and data analyses

Graphs were generated using Prism (GraphPad software). In case of normally distributed data, we determined significance using Student’s t-tests with Bonferroni post hoc corrections in cases of multiple pairwise comparisons. For data that were not normally distributed, we used Mann-Whitney test. We made corrections for multiple hypotheses testing whenever appropriate. Conventions used for describing significance in the figures were: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; n.s., not significant (P > 0.05).

Expanded View for this article is available online.

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Author contributions

JJ performed the majority of the experiments with help from K-JC, AKN, KNC, COW, MV, SG, NEK, LT, HL, MAR, KMT, YZ, and KV. KV performed the bioinformatic analyses. COW helped with Ca²⁺ imaging and cholesterol measurements. KNC, MAR, and KMT helped with Western blot analyses. MV and AGS performed the CAM assay. NEK helped with the generation of shRNA and stable transfections of cancer cells. LT performed some of the cell staining. SG and IL generated and analyzed GPMVs. K-JC, HL, YZ, and JFH performed and supervised the experiments. AKN and DH performed some of the cell proliferation experiments. JJ and KV analyzed the data, and KV wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


TRPML1 allows cancer cell proliferation

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Figure EV1. Correlation between network centrality and membership to the CLEAR family of endolysosomal genes.
A Bar graph showing the relative expression of the indicated genes in the indicated cell types. Bar graphs in the top and bottom panels represent the results of MITF and TFEB knockdown, respectively. Values were normalized to respective HN31 average and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.
B Bar graph showing that the mean closeness centrality of CLEAR target genes is significantly higher than that of genes that do not belong to the CLEAR network. The lines in the bars represent mean values. Statistical test employed was Student’s t-test.
C GSEA conducted on endolysosomal genes ranked on the basis of increasing network centrality. Positions of the CLEAR target genes are indicated.
Figure EV2. Roles for the CLEAR network genes in the proliferation of cancer cells with oncogenic HRAS.

A Graphs showing the relative cell numbers in HN31 cells exposed to the indicated concentrations of siRNAs against MITF and TFEB. All values represent mean ± SEM, n = 3 biological replicates. Statistical tests employed were one-sample t-test to examine significance of differences from a hypothetical value of 1.

B, C Bar graphs showing relative levels of MCOLN1 mRNA in the indicated cells types after the indicated perturbations. Values were normalized to “no knockdown” (B) or respective “control siRNA” (C) and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test. Bonferroni post hoc correction was applied when for multiple pairwise comparisons in (B).

D Bar graph showing relative levels of VAC14 mRNA in HN31 cells after the indicated perturbations. Values were normalized to HN31 average and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test with Bonferroni post hoc correction for multiple pairwise comparisons.

E Line graphs showing relative cell numbers in sample of the indicated genotypes treated with indicated ML-SI1 concentrations. All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test between relative cell numbers at each concentration.

F, G Bar graphs showing the relative cell numbers in the indicated cell types following the indicated treatments. All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.
Figure EV3. TRPML2 and TRPML3 play minor roles in HRAS-driven cancer cell proliferation.

A Violin plots of average Z-scores of the indicated genes in BLCA, THCA, and HNSC patients with oncogenic HRAS mutations. The thick horizontal dashed lines in each sample set represent median; thin dashed lines represent the quartiles, polygons represent density estimates of data and extend to extreme values. Statistical tests employed were 1-sample t-test or Wilcoxon signed rank test to examine significance of differences from a hypothetical value of 0.

B Bar graph showing relative levels of MCOLN1, MCOLN2, and MCOLN3 mRNAs in the indicated cells types. All values were normalized to respective "HRAS shRNA" average and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.

C Bar graph showing relative levels of MCOLN2 and MCOLN3 mRNAs in HN31 cells after the indicated perturbations. Values were normalized to respective HN31 average and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test with Bonferroni post hoc correction for multiple pairwise comparisons.

D Bar graph showing the relative cell numbers in the indicated cell types following the indicated treatments. All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test with Bonferroni post hoc correction for multiple pairwise comparisons.
A) Oncogenic HRAS bearing BLCA, THCA, HNSC

B) MCOLN1, MCOLN2, MCOLN3

C) TMTM1, MCOLN1, MCOLN2, MCOLN3

D) HN31 control shRNA, MCOLN2 shRNA, MCOLN3 shRNA, MCOLN1 siRNA, MCOLN1 shRNA + ML-SI1

Figure EV3.
Figure EV4. TRPML1 and cholesterol are required for the clustering of HRAS.

A Bar graph showing relative levels of MCOLN1 mRNA in the MDCK cells treated with siRNA against MCOLN1. Values are normalized to control average and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.

B The extent of GFP-HRASWT nanoclustering quantified as $L_{max}$ in cells exposed to the indicated perturbations. Values were normalized to DMSO average and represent mean ± SEM ($n \geq 15$ for each condition). Statistical significances of differences were evaluated in bootstrap tests.

C Bar graph showing GFP-HRASWT content in the inner leaflet of the plasma membrane is quantified as the number of gold particles per 1 µm$^2$ region from electron micrographs. Values were normalized to DMSO average and represent mean ± SEM, $n \geq 15$ for each condition. Statistical test employed was ANOVA.

Figure EV5. Drugs that affect cellular cholesterol levels impact growth of cancer cells with oncogenic HRAS.

A, C Graphs showing relative cell numbers indicated cell types following application of β-cyclodextrin (A) or statin (C). Cell numbers were assessed after a 24-h period of growth in cultures and normalized to the values obtained in the absence of drug. Values represent mean ± SEM, $n \geq 3$ biological replicates.

B Left, graph showing that the relative cell numbers of concentration of β-cyclodextrin could be fit to a Hill input function for repressor with Hill coefficient = 4. Right, bar graph showing $K_i$ values in the indicated cell types. Values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.

D Same as the bar graph in (B), but in case of cells treated with statins.

E Confocal images of control or ML-SI1-treated cells that were exposed to recombinant GFP-lysenin. Intracellular GFP-lysenin punctae indicate that ML-SI1 led to the internalization of sphingomyelin from the plasma membrane into intracellular vesicles. Scale bar (10 µm) shown in the panel on the top right applies to all panels.
**A**

HN31 cells

**B**

HN31 cells
HRAS shRNA

**C**

HN31 cells

**D**

HN31 cells
HRAS shRNA

**E**

GFP-lysennin

DAPI

merge

Figure EV5.
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Appendix Figure S1. List of genes that belong to the clusters shown in Fig 1. The order of the genes in each cluster is the order in which those genes appear on the dendrogram. GGH and APCS do not belong to any of the clusters.
Appendix Figure S2. (A-B) Bar graphs showing fraction (A) and total number (B) of Ki67 labeled nuclei in HN31 cells exposed to DMSO or the indicated concentrations of ML-S11. Times indicated below the graphs are the number of days after the initial implantation of the cells. All values represent mean±SEM. Data points represent values from independent eggs. Statistical test employed was Student’s t-test. Values in (B) were normalized to respective DMSO average.

(C) Bar graph showing relative levels of MCOLN1 mRNA in the UM-SCC-22A cells expressing HRAS\textsuperscript{WT} or HRAS\textsuperscript{G12V}. Values were normalized to HRAS\textsuperscript{WT} average and represent mean±SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.

(D) Kaplan-Meier curves showing the survival of patients stratified on the basis of MTM1 expression in BLCA and HNSC patients whose tumors were wild-type for HRAS (right) or carried oncogenic HRAS mutations (left).

(E) Kaplan-Meier curves showing the survival of patients stratified on the basis of MCOLN1 expression in YHCA patients whose tumors were wild-type for HRAS (right) or carried oncogenic HRAS mutations (left). Statistical tests employed in (D-E) were Mantel-Cox log-rank test.
Appendix Figure S3. (A-B) Representative Western blots generated using extracts from MDCK cells expressing GFP-tagged oncogenic HRAS and controls treated with DMSO or ML-SI1 as indicated. The primary antibodies used were against GFP (A) and HRAS (B). Values were normalized to respective DMSO average and represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.

(D) Representative Western blots generated using extracts from HN31 cells exposed to the indicated perturbations. Primary antibodies used are indicated.

(E) Bar graph showing quantification of the Western blots shown in (D). All values represent mean ± SEM. Data points represent values from biological replicates. Statistical test employed was Student’s t-test.
Appendix Figure S4. Bar graph showing pERK/ERK quantification of the Western blots shown in Fig 5D. Values represent mean±SEM. Data points represent values from biological replicates. Statistical tests employed were Student’s t-test.