

## ORIGINAL ARTICLE

# Cowden syndrome-associated germline succinate dehydrogenase complex subunit D (SDHD) variants cause PTEN-mediated down-regulation of autophagy in thyroid cancer cells

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## Abstract

Thyroid cancer is a major component cancer of Cowden syndrome (CS), a disorder typically associated with germline mutations in *PTEN*. Germline variants in succinate dehydrogenase genes (*SDHx*) co-occurring with *PTEN* germline mutations confer a 2-fold increased prevalence (OR 2.7) of thyroid cancer compared to *PTEN*-associated CS but 50% decreased prevalence (OR 0.54) of thyroid cancer compared to *SDHx*-associated CS. We have previously shown that CS-associated *SDHD* variants G12S and H50R induce *PTEN* oxidation and nuclear accumulation in thyroid cancer. Our current study shows that *SDHD*-G12S and -H50R variants cause down-regulation of autophagy, demonstrating a role for *SDHD* in autophagy-associated pathogenesis of differentiated thyroid cancer. These findings could explain the increased prevalence of thyroid cancer in CS patients with *SDHx* germline mutations compared to those with *PTEN* mutations alone. Importantly, we demonstrate the dependence of this process on functional wild-type *PTEN* with reversal of decreased autophagy after *PTEN* knockdown. The latter could explain the clinically observed decrease in thyroid cancer prevalence in patients with co-existent *PTEN* mutations and *SDHx* variants. We also show that *SDHD*-G12S/H50R promotes mono-ubiquitination of *PTEN*, causing its translocation into the nucleus, upregulation of AKT and consequent phosphorylation of FOXO3a. Furthermore, *SDHD*-G12S/H50R-mediated increase in acetylation of FOXO3a further enhances AKT-associated phosphorylation of FOXO3a. This combination of phosphorylation

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and acetylation of FOXO3a results in its nuclear export for degradation and consequent down-regulation of FOXO3a-target autophagy-related gene (ATG) expression. Overall, our study reveals a novel mechanism of crosstalk amongst SDHD, PTEN and autophagy pathways and their potential roles in thyroid carcinogenesis.

## Introduction

Thyroid cancer in the general population has been increasing in incidence over the last 10 years, without clear etiology but likely in part related to increased detection. It is also being increasingly recognized that thyroid cancer can often occur in families, including in inherited neoplasia syndromes such as Cowden syndrome (CS). The thyroid is one of the major organs affected in CS with pathologies including follicular thyroid adenomas, thyroid hamartomas, Hashimoto thyroiditis and differentiated thyroid carcinoma. CS is a difficult-to-detect, under-diagnosed autosomal dominant inherited disorder characterized by multiple hamartomas affecting derivatives of all three germ layers and a high risk of solid tumors, such as those of the thyroid, breast and kidney. Germline mutations in the gene encoding phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor are found in approximately 25% of classic CS patients and CS-like patients, combined (1). Germline variants of genes encoding the different subunits of succinate dehydrogenase (SDHB-D) have been found (and subsequently validated) in ~10% of PTEN mutation negative CS/CS-like individuals (2,3). Notably, SDHB-D variants can co-exist with in germline PTEN mutations in approximately 6–8% of CS/CSL individuals and appear to modify the risk of both breast and thyroid cancer (2).

Succinate dehydrogenase (SDH) or complex II of the mitochondrial respiratory chain is a unique membrane-bound enzyme that plays dual functions in both the Krebs cycle for succinate oxidation and the respiratory chain for electron transfer to the terminal acceptor ubiquinone. SDH is a highly conserved hetero-tetrameric protein, with SDHA and SDHB as catalytic subunits that protrude into the mitochondrial matrix and SDHC and SDHD as structural anchors on the inner membrane. Each of the genes encoding the four subunits of SDH (SDHA-D) is considered to be a classic tumor suppressor gene (4). Germline SDHB-D variants have been shown to upregulate the AKT and MAPK pathways, similar to that seen in PTEN dysfunction (3). Compared to PTEN mutation carriers, SDHx variant carriers have significantly higher frequencies of breast, thyroid, and renal cell carcinomas (2,3). The two most common SDHx variants in CS/CS-like individuals are SDHD-G12S and SDHD-H50R (2,3). We have recently shown that SDHD-G12S and SDHD-H50R result in upregulation of reactive oxygen species (ROS) resulting in oxidized PTEN (thus, loss-of function) which accumulates in the nucleus in follicular thyroid cancer cell lines (5). However, the functional interactions between the SDH and PTEN pathways are not completely understood.

Accumulating evidence supports the critical role of autophagy in cancer development from initiation to progression and metastasis, including in thyroid carcinoma. Autophagy is a catabolic process present in all eukaryotic cells, involving degradation of the cell's own components through the lysosomal machinery. Basal levels of autophagy are fundamental to maintaining cellular homeostasis and play an integral part in cell growth and development. Inability of the cell to up-regulate autophagy, for instance due to defective autophagy-related genes (ATGs), can result in accumulation of defective molecules or excessive ROS, which in turn impair cellular functions and DNA stability resulting in cancers (6). Whether autophagy negatively or positively regulates the

development of thyroid cancer is not clear yet (7). The paradoxical role of autophagy in prevention of cell transformation or promotion of cancer cell survival depends on its regulation by various signaling pathways, like the MAPK and PI3K/AKT pathways. Based on these known pathways for autophagy and previous observations by our group stated above, we sought to address our hypothesis that CS-associated SDHD variants (G12S, H50R) cross-talk with the PTEN pathway to influence autophagy and hence predispose thyroid cells to carcinogenesis.

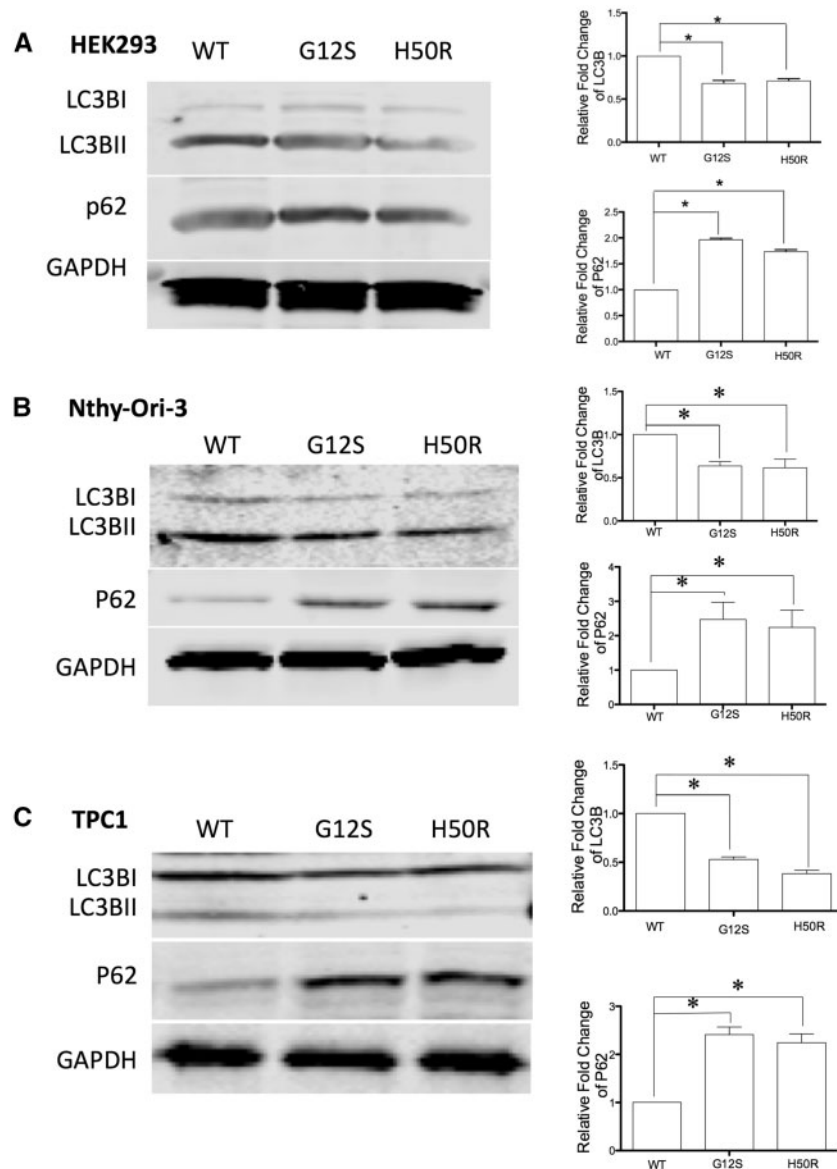
## Results

### Cell lines expressing SDHD-G12S, or -H50R variants show increased cell growth compared to SDHD-wildtype-expressing cells

Nthy-Ori-3.1 'normal' thyroid follicle and TPC1 papillary thyroid cancer cell lines stably expressing Flag-tagged SDHD-WT, -G12S, or -H50R constructs were analyzed. Growth curves obtained via daily cell counts indicate that SDHD-G12S or -H50R expressing Nthy-Ori-3.1 and TPC1 cells grow faster than SDHD-WT cells (Supplementary Material, Fig. S1A and B). We observed that equal numbers of plated Nthy-Ori-3.1 cells expressing SDHD-G12S or SDHD-H50R were completely confluent on day 5 and 6 of culture, respectively, whereas SDHD-WT expressing cells showed only 50% confluence by day 6. Similar results were observed in TPC1 cells where cell numbers of the SDHD-G12S or -H50R expressing cell lines were double that of SDHD-WT expressing cells on day 5 of culture.

### SDHD-G12S or -H50R variants suppress autophagy in HEK293, Nthy-Ori-3.1 and TPC1 cells

To investigate if SDHD variants affect autophagy, two autophagy markers were assessed by Western blotting. LC3B is an ubiquitin-like protein that is necessary for the formation of autophagosomes. It is recruited from the cytosol and associated with autophagophores during the process of autophagy. With increased levels of autophagy, LC3B I (16KD) is cleaved into LC3B II (14KD) (8). Therefore, increased expression levels of LC3B II can serve as a marker for increased autophagy. In contrast, p62 is an ubiquitin-binding scaffold protein degraded by autophagy. Since p62 accumulates when autophagy is inhibited, decreases in p62 levels can serve as a marker for increased induction of autophagy (9). Western blot analysis of lysates from HEK293, Nthy-Ori-3.1 and TPC1 cells with stable expression of SDHD-G12S or SDHD-H50R constructs showed decreased LC3B II levels and increased p62 accumulation compared to the SDHD-WT expressing cells (Fig. 1A–C, respectively). No increase in p62 levels was observed in SDHD-G12S or SDHD-H50R expressing cells compared to SDHD-WT expressing cells (Supplementary Material, Fig. S2). Cyto-ID staining for vesicles produced during autophagy showed more intense vesicle staining (green) in SDHD-WT cells than that in the cell lines expressing SDHD-G12S or SDHD-H50R (Fig. 2). Using three experimental read-outs, our data demonstrate that SDHD-G12S and SDHD-H50R variants



**Figure 1.** SDHD-G12S or -H50R inhibit autophagy in HEK293, Nthy-Ori-3.1 and TPC1 cells. Western blot for LC3BII and p62 autophagy markers in (A) HEK293, (B) Nthy-Ori-3.1 and (C) TPC1 cells stably transfected with SDHD-WT, SDHD-G12S or SDHD-H50R. GAPDH was blotted as loading control (left panels). Note that quantification of intensity of LC3BII and p62 was normalized to GAPDH from three independent experiments (\* indicates  $P < 0.05$  and \*\*\* indicates  $P < 0.01$ ) (right panels).

decrease autophagy levels in HEK293 cells and in 'normal' follicular as well as malignant thyroid cell lines.

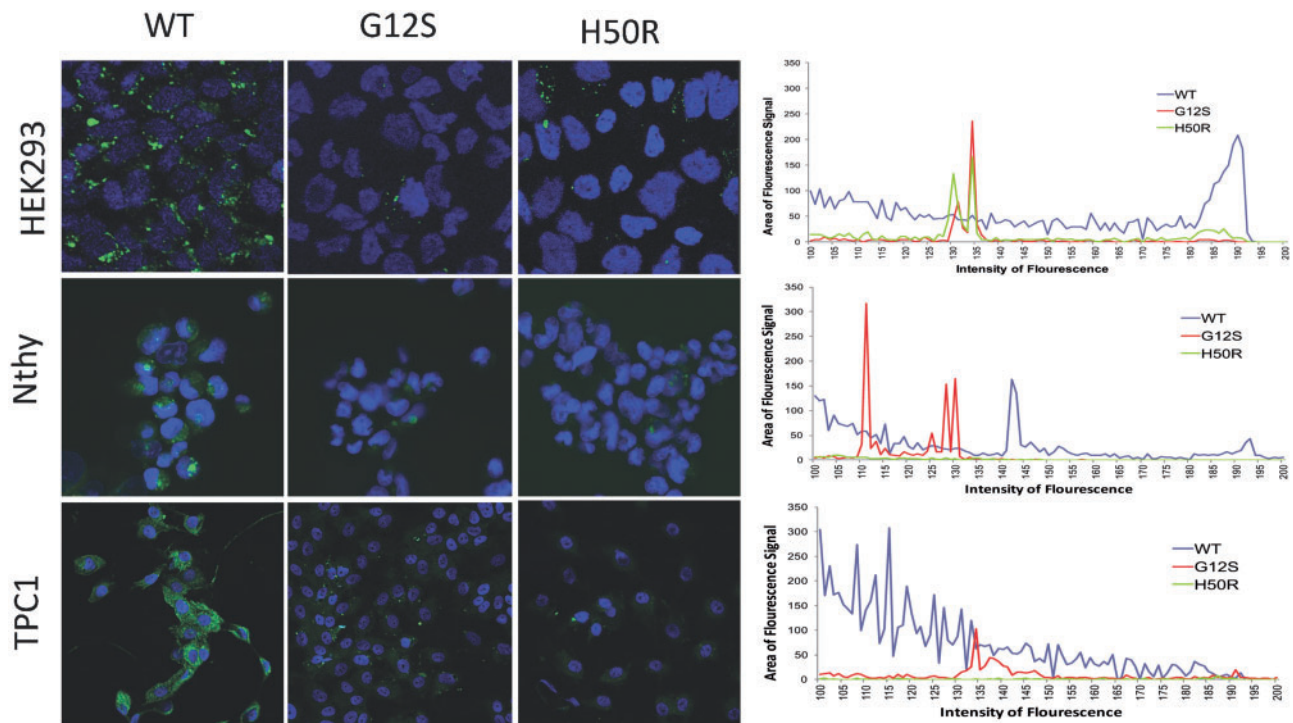
#### SDHD-G12S/H50R variants induce degradation of FOXO3a

As a master regulator of autophagy, FOXO3a expression was investigated and compared between SDHD-WT-expressing cells and SDHD-G12S or SDHD-H50R-expressing cells in HEK293, Nthy-Ori-3.1 and TPC1 lines. In all three cell lines, Western analysis showed that FOXO3a levels decreased in cells expressing SDHD-G12S or SDHD-H50R compared to SDHD-WT expressing cells (Fig. 3, left panels). Since protein levels of FOXO3a are mainly regulated by proteasome-mediated degradation, we next investigated the ubiquitination status of FOXO3a in all stable cell lines. We

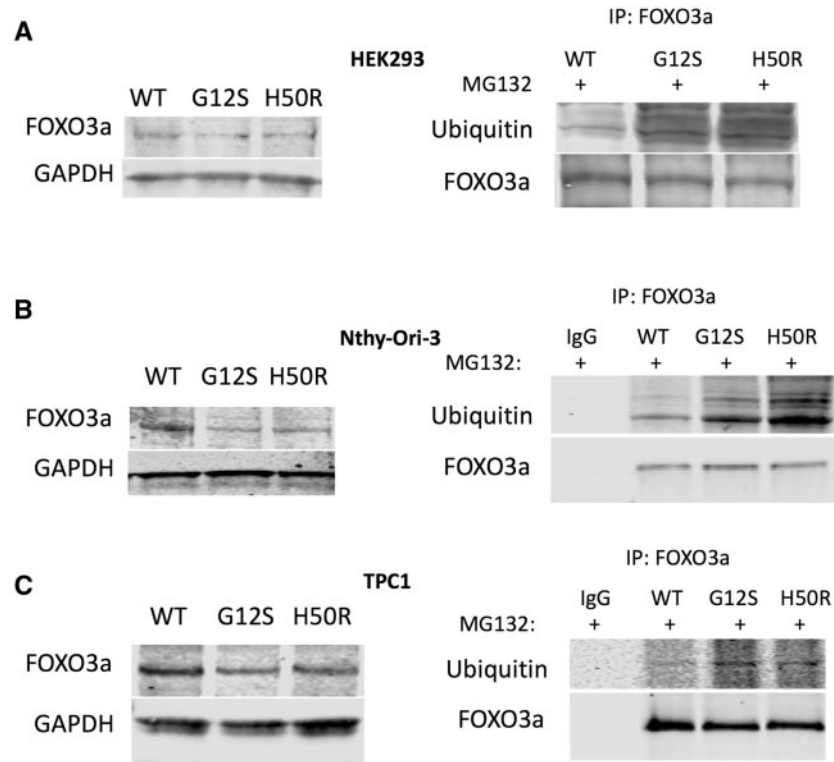
found that ubiquitination of FOXO3a increased in SDHD-G12S and SDHD-H50R expressing cells compared with that in SDHD-WT-expressing cells across all the three cell lines (Fig. 3, right panels), suggesting that SDHD-variants may down-regulate FOXO3a through an increase in its ubiquitination.

#### SDHD-G12S/H50R variants promote phosphorylation of nuclear FOXO3a

Localization of FOXO3a is critical for its activity and is determined by its phosphorylation levels (10). Upon AKT-mediated phosphorylation at Serine-253, phosphorylated FOXO3a is translocated to the cytoplasm and subsequently binds to 14-3-3 leading to its degradation by proteasomes (10). In contrast, dephosphorylation of FOXO3a by PP2A, facilitates FOXO3a

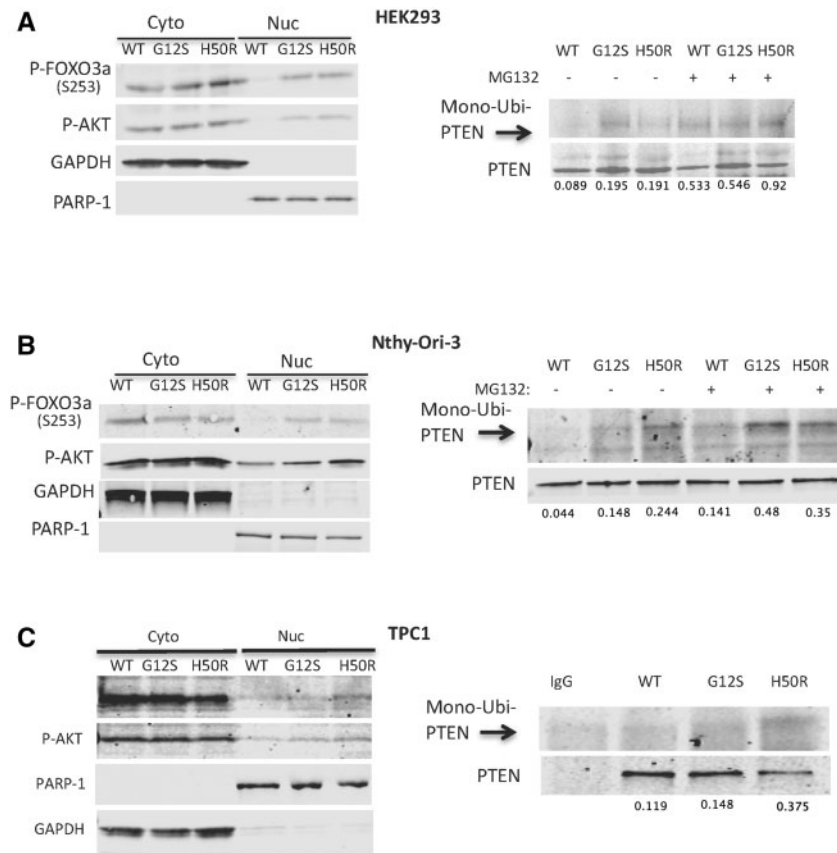


**Figure 2.** SDHD-G12S or -H50R inhibit autophagy vesicle formation in HEK293, Nthy-Ori-3.1 and TPC1 cells. Cyto-ID staining (Green) for autophagosomes reveals increased staining in HEK293, Nthy-Ori-3.1 and TPC1 cells expressing SDHD-WT (left panels) compared to those expressing SDHD-G12S (middle panels) or -H50R (right panels). Blue staining represents nuclei. Comparative quantification of Cyto-ID staining (Green) is shown as a histogram of signal intensity (X-axis) vs signal area (Y-axis) for SDHD-WT (Blue), SDHD-G12S (Red) and SDHD-H50R (Green) expressing cells. Magnification for all panels:  $10 \times 40$ .



**Figure 3.** SDHD-G12S or SDHD-H50R decrease FOXO3a protein levels through proteasome-mediated degradation in HEK293, Nthy-Ori-3 and TPC1 cells. Western blot for total FOXO3a expression (left panels) in HEK293 (A), Nthy-Ori-3 (B) and TPC1 (C) and Immunoprecipitation (IP) Western blots for FOXO3a-ubiquitination in the corresponding cell lines treated with proteasome inhibitor MG132 (right panels). Blots shown are best representatives of three separate experiments.





**Figure 4.** SDHD-G12S or -H50R induce PTEN mono-ubiquitination accompanied by increased nuclear phosphorylation of FOXO3a and AKT. Western analyses for phosphorylation of FOXO3a or AKT in nuclear and cytoplasmic fractions from HEK293 (A), Nthy-Ori-3 (B) and TPC1 (C). GAPDH and PARP-1 were used as cytoplasmic and nuclear loading controls, respectively (left panel). Assay for mono-ubiquitination of PTEN was performed by PTEN immunoprecipitation followed by Western blot for ubiquitin in the presence (+) or absence (-) of MG132 in the context of stable expression of either SDHD-WT or SDHD-G12S/H50R (right panels). Numbers below each set of bands represents the intensity ratio of mono-ubiquitinated PTEN to total PTEN, the latter as loading control. All blots shown are best representatives of three separate experiments.

relocation into the nucleus, thereby activating its target genes (10). Therefore, we analyzed FOXO3a protein localization through subcellular fractionation followed by Western blot in the cell lines expressing SDHD-WT, SDHD-G12S, or SDHD-H50R. Although cytosolic FOXO3 phosphorylation was similar between the three transfectants in each cell line, in all cases, elevated nuclear FOXO3a phosphorylation was observed in the SDHD-G12S and SDHD-H50R-expressing cells compared to SDHD-WT (Fig. 4, left panels).

In addition, we observed increased levels of phosphorylated AKT in the nucleus of all three cell lines expressing SDHD-G12S or -H50R compared to those expressing SDHD-WT (Fig. 4, left panels). Since PTEN is a major negative regulator of AKT, we assessed total PTEN protein levels by Western blot but found no difference between cells expressing SDHD-WT and those expressing SDHD-G12S/H50R (data not shown). Previous studies have reported that PTEN nuclear import is mediated by its mono-ubiquitination (11). To explain increased phosphorylation of nuclear AKT, we measured mono-ubiquitination of PTEN. Indeed, we observed increased mono-ubiquitinated PTEN in HEK293, Nthy-Ori-3.1 cells and TPC1 cells expressing SDHD-G12S or -H50R but not in SDHD-WT expressing cells (Fig. 4, right panels).

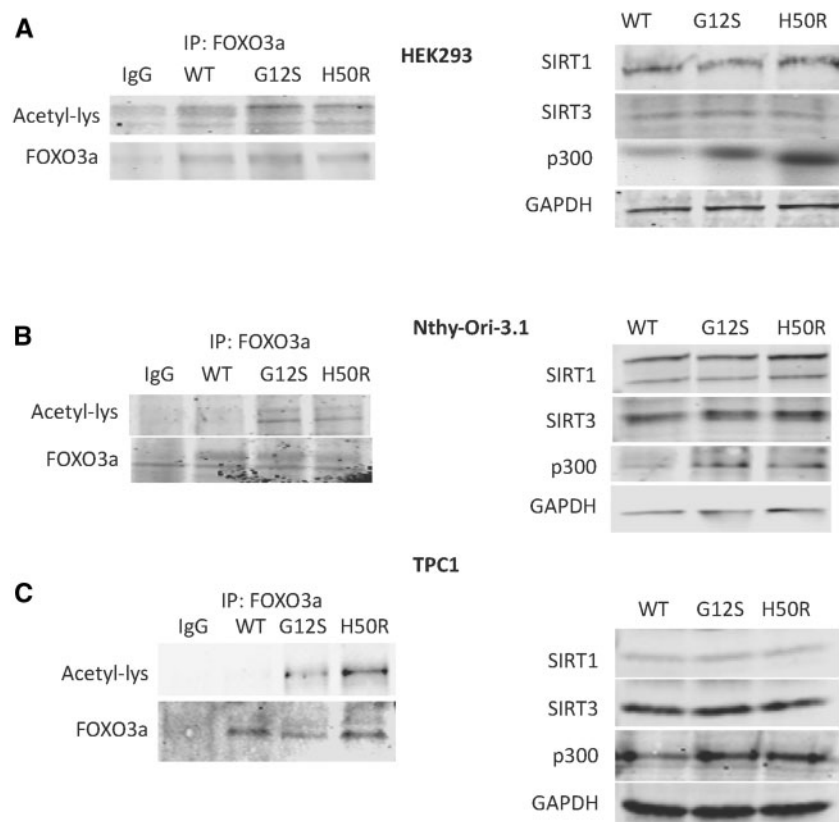
Taken together, our data suggest that SDHD-G12S or SDHD-H50R cause(s) mono-ubiquitination of PTEN, with consequent

translocation into the nucleus accompanied by upregulation of AKT and further promotes phosphorylation of nuclear FOXO3a (also, see below).

#### SDHD-G12S/H50R variants promote acetylation of nuclear FOXO3a

It is accepted that the acetylation status of FOXO transcription factors influence transcriptional activity (10). To investigate if SDHD variants affect FOXO3a acetylation, immunoprecipitation of FOXO3a with Acetyl-Lys was performed in HEK293, Nthy-Ori-3 and TPC1 cells expressing SDHD-WT, SDHD-G12S or SDHD-H50R. Augmented acetylation of FOXO3a was observed in all three cell lines expressing SDHD-G12S or SDHD-H50R compared to their corresponding SDHD-WT-expressing cells (Fig. 5, left panels).

Since the SIRT1 deacetylase and p300 acetyltransferase are responsible for the acetylation status of FOXO transcription factors (12–14), we investigated whether the SDHD variants affect the expression of these two enzymes. Ectopic expression of SDHD-G12S or SDHD-H50R in HEK293, Nthy-Ori-3 and TPC1 cell lines is accompanied by an increased p300 expression but no decrease of SIRT1 compared to cells expressing SDHD-WT (Fig. 5, right panels). These observations suggest that SDHD



**Figure 5.** SDHD-G12S or SDHD-H50R increases FOXO3a acetylation through up-regulation of p300. Acetylation of FOXO3a was assayed by immunoprecipitation of FOXO3a followed by anti-Lysine-acetylation Western blot in HEK293 (A), Nthy-Ori-3 (B) and TPC1 (C) cells expressing SDHD-WT, SDHD-G12S or SDHD-H50R (left panels); Western blot for acetyl-transferase (p300) and two relevant deacetyltransferases (SIRT1, SIRT3) were performed in the three corresponding cell lines (right panels). Blots shown are best representatives of three separate experiments.

variants induce FOXO3a acetylation in association with up-regulation of expression of p300-acetyltransferase.

#### SDHD-G12S/H50R variants down-regulate expression of FOXO3a target genes

To further investigate the effect of SDHD variants on FOXO3a activity, mRNA and protein expressed from two 'read out' FOXO3a target genes, *BECN1* and *ATG12*, were analyzed. In HEK293 and TPC1 cells, decreased *BECN1* and *ATG12* mRNA levels were observed in SDHD variant-expressing cells compared to SDHD-WT-expressing cells (Fig. 6A and C, left panel). In Nthy-Ori-3 cells, we observed decreased *BECN1*, but not *ATG12*, mRNA levels in SDHD-variant expressing cells compared to SDHD-WT controls (Fig. 6B, left Panel). Notably, expression of SDHD-G12S or H50R, but not SDHD-WT, in each of the three cell lines, HEK293, Nthy-Ori-3 and TPC1, showed decreased *BECLIN1* and *ATG12* protein expression by Western analysis (Fig. 6A–C, right panels). Taken together, our observations suggest that SDHD-G12S and -H50R variants suppress FOXO3a activity resulting in decreased expression of autophagy-related genes.

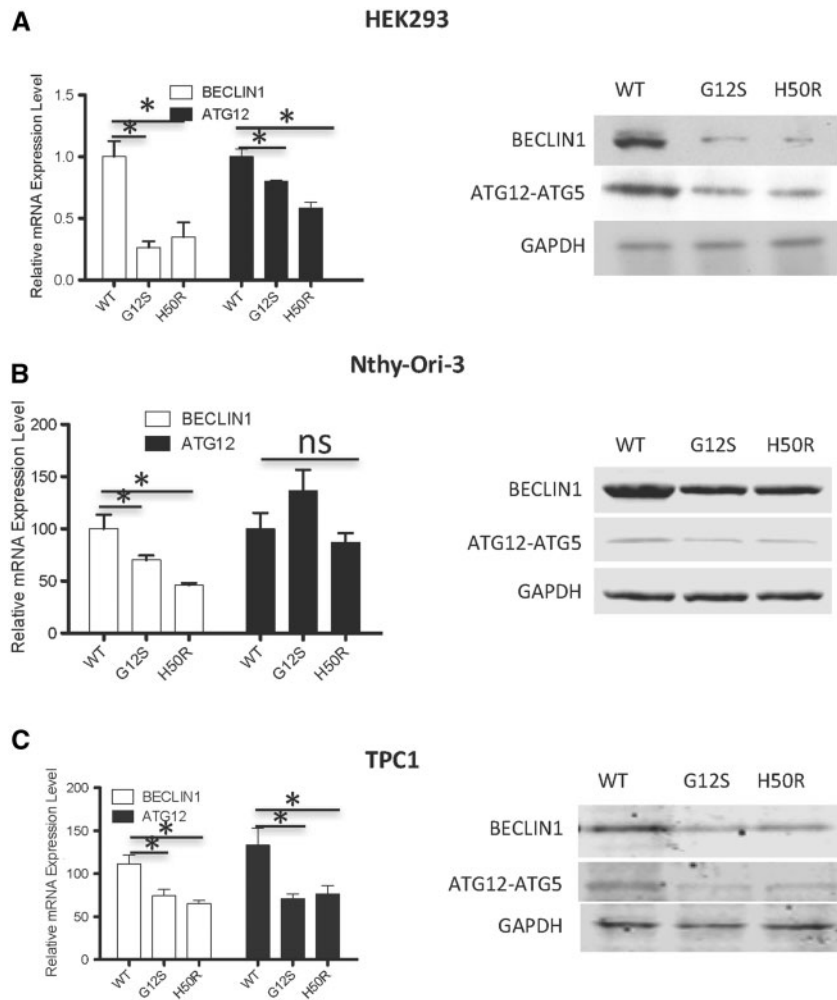
#### PTEN is necessary for SDHD variant-associated inhibition of autophagy

To further examine if PTEN is required for SDHD variant-associated autophagy inhibition, PTEN was knocked down in

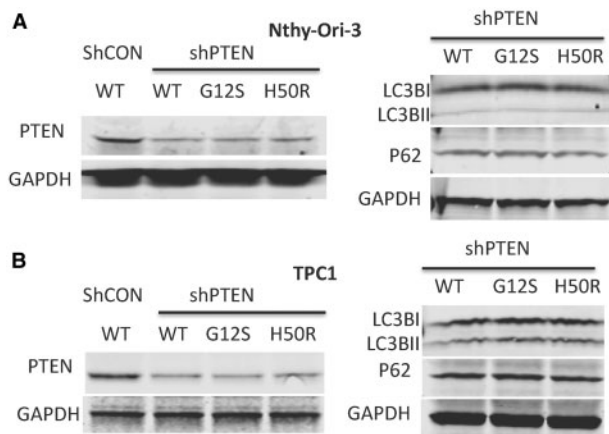
Nthy-Ori-3 and TPC1 cells transfected with SDHD-WT, SDHD-G12S or SDHD-H50R (Fig. 7, left panels). Western blots for autophagy markers LC3B and p62 were performed to determine autophagy levels. The SDHD-G12S/H50R-mediated inhibition of autophagy was abrogated when PTEN was knocked down (Fig. 7, right panels). These data suggest that PTEN expression is necessary for SDHD-variant-associated autophagy suppression.

#### Discussion

It has been established that germline SDHD variants and somatic SDHD/B alterations are associated with heritable and sporadic thyroid cancer, respectively (15–17). We have also demonstrated previously that SDHD variants promote apoptosis in follicular thyroid cancer cells (5). Apoptosis and autophagy are intricately linked processes where modulation of autophagy controls increase or decrease in apoptosis as per the requirements of the cell to survive or perish (18,19). In this study, we show data confirming that SDHD variants regulate autophagy in both benign and malignant thyroid cells, suggesting that this function may be involved in SDH variant-associated thyroid cancer. Here, we show that expression of SDHD-G12S and SDHD-H50R lead to increased nuclear phosphorylation of AKT, and subsequent phosphorylation of the transcription factor FOXO3a, the master regulator of autophagy. It has been shown that AKT-mediated phosphorylation at Ser-253 on FOXO3a, leads to its cytoplasmic localization and degradation by proteasomes (20). In addition, we observed that SDHD variants



**Figure 6.** SDHD-G12S or SDHD-H50R downregulates FOXO3a autophagy-related target gene expression. qRT-PCR for FOXO3a target genes BECLIN1 and ATG12 in HEK293 (A), Nthy-Ori-3 (B) and TPC1 (C) cells expression SDHD-WT, SDHD-G12S or SDHD-H50R (\* indicates  $P < 0.05$ ) (left panels); Western blots for BECLIN1 and ATG12 in the three corresponding cell lines (A-C, right panels).



**Figure 7.** SDHD-G12S or SDHD-H50R suppressing autophagy is PTEN-dependent. Western blot showing PTEN knockdown in Nthy-Ori-3 (A) and TPC1 (B) thyroid cells with stable expression of SDHD-WT or SDHD variants G12S or H50R (left panels). Western blot reveals autophagy marker (LC3BII and p62) levels in (A) Nthy-Ori-3 and (B) TPC1 cells stably expressing SDHD-WT, SDHD-G12S or SDHD-H50R after PTEN knockdown (right panels). Blots shown are best representatives of three separate experiments.

increased acetylation of FOXO3a in association with p300 overexpression. Therefore, phosphorylation and acetylation of FOXO3a together lead to its nuclear export and binding to 14-3-3 for degradation. This ultimately results in suppression of autophagy via down-regulation of FOXO3a autophagy-related, target genes such as BECN1 and ATG12 (Fig. 8).

There is increasing evidence of crosstalk between PTEN and mitochondrial signaling pathways, but the precise mechanism of how dysfunctional mitochondrial pathways or mutations in mitochondrial tumor suppressor genes such as SDHx lead to PTEN dysfunction, and result in neoplastic transformation is not fully understood. Our current observations elucidate a novel mechanism that links CS-associated SDHD variants (SDHD-G12S, SDHD-H50R) to PTEN function. In addition to inducing oxidized PTEN, as suggested by our earlier studies, we now show that SDHD-G12S or -H50R stimulate mono-ubiquitination of PTEN, which leads to PTEN dysfunction with consequent upregulation of pAKT, especially of nuclear pAKT. Notably, we observed that this inhibition of autophagy by SDHD variants -G12S or -H50R was dependent on functional PTEN (Fig. 7). Although we recognize that our data are limited to cell lines, our observation that knocking down PTEN abrogates SDHD-G12S or -H50R suppressed autophagy might explain a puzzling clinical genetic

observation in CS/CS-like individuals. The lifetime risk of epithelial thyroid cancer in *PTEN* mutation positive persons is 35% (21). Our initial observations of evaluable CS/CS-like research participants revealed an odds ratio (OR) of three (95% CI 1.47–6.19;  $P < 0.002$ ) for thyroid cancer in individuals with *SDHx* variants compared to those with *PTEN* mutations alone (2). Individuals with both *PTEN* mutations and *SDHx* variants have an OR 0.34 (0.12–1.08;  $P = 0.038$ ) compared to those with *SDHx* variants alone, and an OR 1.08 (0.39–3.05;  $P = 0.21$ ) compared to those with germline *PTEN* mutations alone (2). We have subsequently accrued an independent validation series. Compared to *PTEN* mutation only, OR for thyroid cancer was 4.95 in CS/CSL with *SDHx* variants only ( $P < 0.001$ ) and 2.70 for those carrying *PTEN* mutation and *SDHx* variant ( $P = 0.003$ ; Table 1). Compared to *SDHx* variants only, OR for thyroid cancer for those carrying both *PTEN* mutation and *SDHx* variant was 0.54. *SDHx*-variant-associated suppression of autophagy could help explain the increased risk of thyroid cancers in our CS/CS-like patients with *SDHx* variants. Our current observation that *PTEN* knockdown reverses/rescues *SDHx*-variant-associated autophagy suppression might help explain the decreased prevalence of thyroid cancers when both *PTEN* mutation and *SDHx* variants co-exist compared to those with only *SDHx* variants.

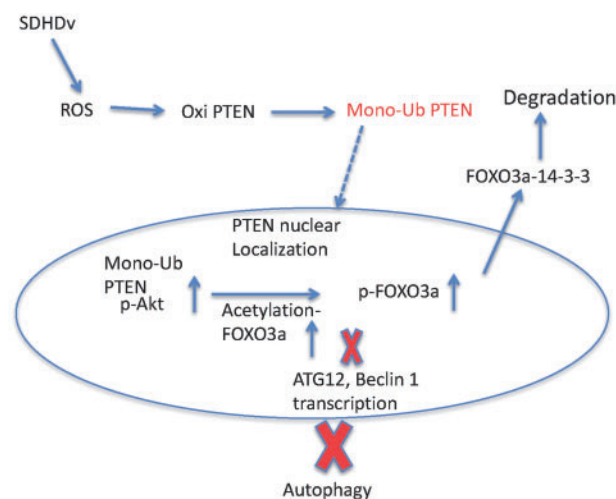


Figure 8. Schematic diagram depicting the signaling pathway for the germline *SDHD* variants affecting autophagy via *PTEN* mono-ubiquitination.

That *SDHD*-G12S/H50R-mediated suppression of autophagy requires wildtype (WT) *PTEN* suggests that *SDHD* variants increase phosphorylation of nuclear AKT only when intact *PTEN* is present, and that nuclear AKT is activated by mono-ubiquitinated *PTEN*. The important role of nuclear AKT in multiple cellular processes such as apoptosis, cell cycle, DNA repair, cell differentiation and tumorigenesis are well known (22–24). Previous work from our laboratory demonstrated that *PTEN* is necessary and sufficient for inhibiting AKT activation in the nucleus through its intact lipid phosphatase activity and proper subcellular localization (25). Ubiquitination of *PTEN* inhibits its activity (26). Mono- and poly-ubiquitination appear to have separate effects on *PTEN* function, with mono- and poly-ubiquitination targeting *PTEN* for nuclear localization and proteasome degradation, respectively. In the current study, we show that *SDHD* variants G12S and H50R induce mono-ubiquitination of *PTEN*, which translocates into the nucleus, where it functions as a dominant negative *PTEN*, resulting in AKT upregulation and subsequent tumorigenesis. Overall ubiquitination status of *PTEN* is determined by both ubiquitination and de-ubiquitination. Many E3 ligases, such as NEDD4-1, WWP2, XIAP and RFP, and de-ubiquitylase, such as HAUSP/USP7, OTUD3, and USP13, have been identified (27). XIAP (X-Linked Inhibitor of Apoptosis), an anti-apoptotic protein, is involved in mono- and poly-ubiquitination of *PTEN* and knock-down of XIAP prevented nuclear translocation of *PTEN* (28). Though XIAP is a good candidate E3 ligase for *SDHD* variant-mediated mono-ubiquitination of *PTEN*, identification of the precise E3 ligase needs to be further investigated.

Our current data show increased phosphorylation of nuclear FOXO3a at S253 in *SDHD*-G12S/H50R transfected cells in association with an increase in phosphorylation of nuclear AKT. AKT is an important kinase downstream of *PTEN* and that directly phosphorylates FOXO3a at T32, S253 and S315 (29). S253 is the crucial residue regulating the nuclear-cytoplasmic shuttling of FOXO3a. As a transcription factor, FOXO3a is regulated by post-translational modifications such as phosphorylation, acetylation and ubiquitination (30). Phosphorylation of FOXO3a prevents its localization in the nucleus and induces its interaction with 14-3-3 nuclear export protein, resulting in FOXO3a's degradation and functional inactivation by the proteasome pathway. This is consistent with our observation of lower total FOXO3a protein level and elevated ubiquitination of FOXO3a in *SDHD*-G12S/H50R expressing cells. As a generic E3 ligase for FOXO protein, MDM2 is likely involved in ubiquitination of FOXO3a (31). In addition, we observed the two CS-associated *SDHD* variants

Table 1. Comparison of cancer frequencies of adult *SDHx* variant carriers to *PTEN* mutation carriers (Fisher's two-tailed exact test)

		Female Breast Cancer (/total number)	Thyroid Cancer (/total number)	Renal Cancer (/total number)
	<i>PTEN</i> <sup>MT+</sup>	111/302 (36.7%)	65/551 (11.8%)	29/551 (5.2%)
	<i>SDH</i> <sup>MT+</sup>	70/115 (60.8%)	53/133 (39.8%)	21/133 (15.8%)
	<i>PTEN</i> <sup>MT+</sup> / <i>SDH</i> <sup>MT+</sup>	29/46 (63.0%)	17/64 (26.6%)	1/64 (1.5%)
<i>SDH</i> <sup>MT+</sup> vs <i>PTEN</i> <sup>MT+</sup>	P-value	<0.001	<0.001	<0.001
	Odds Ratio (95% CI)	2.67 (1.68, 4.26)	4.95 (3.14, 7.82)	3.375 (1.78, 6.38)
<i>PTEN</i> <sup>MT+</sup> / <i>SDH</i> <sup>MT+</sup> vs <i>SDH</i> <sup>MT+</sup>	P-value	0.86	0.08	0.003
	Odds Ratio (95% CI)	1.09 (0.51, 2.36)	0.54 (0.27, 1.10)	0.10 (0.01, 0.61)
<i>PTEN</i> <sup>MT+</sup> / <i>SDH</i> <sup>MT+</sup> vs <i>PTEN</i> <sup>MT+</sup>	P-value	<0.001	0.003	0.35
	Odds Ratio (95% CI)	3.93 (1.48, 5.87)	2.70 (1.40, 5.18)	0.29 (0.01, 2.01)



(SDHD-G12S, SDHD-H50R) inducing FOXO3a acetylation associated with up-regulation of the acetylase P300. Thus, it seems likely that the deacetylase SIRT1/3 is not involved in the process of acetylation of FOXO3a in the presence of the two SDHD variants.

The role of autophagy in cancer development, including thyroid cancer, is complicated where it may act as a cancer suppressor or promoter, depending on the cancer type and stage of progression (32–36). Modulation of autophagy has been suggested as an effective treatment modality against tumors and is also highly dependent on the type of tumor and its mutation status (35). We observed impaired autophagy in 'normal' or non-transformed thyroid follicular cells (Nthy-Ori-3.1) and papillary thyroid cancer cells (TPC1) when overexpressing either SDHD variant, highlighting the suppressive role of autophagy in cancer development. Our results are in agreement with the model that autophagy exerts its tumor suppressive protective effects during cancer initiation promoted by conditions of stress or oncogene activation. In contrast, once cancer is established, activation of autophagy promotes tumor development by helping the cancer cells meet energy requirements in unfavorable environments. However, even at this stage, excessive activation of autophagy can lead to cell death (18,19,36,37). Our findings on reduced autophagy levels as a result of germline SDHD variants, provide a potential transformation mechanism linking host genetic background to aberrant autophagy levels and predisposition to cancer. The novel mechanistic link between SDHx variants, autophagy and PTEN status in thyroid carcinomas described here provides insights that may impact thyroid cancer diagnosis, prevention, and treatment in heritable and potentially sporadic settings.

## Materials and Methods

### Cell culture

HEK293 embryonic kidney cells were grown in DMEM plus 10% fetal bovine serum at 37°C with 5% CO<sub>2</sub>. Nthy-Ori-3 thyroid follicle cells were grown in RPMI supplemented with 10% fetal bovine serum and 2mM Glutamine and incubated at 37°C with 5% CO<sub>2</sub>. HEK293T cell line was originally purchased from the American Type Culture Collection (ATCC, Manassas, VA) in 2011 and obtained in 2014 from the Cleveland Clinic Lerner Research Institute Cell Culture Core. The Nthy-ori 3-1 human thyroid follicular epithelium cell line (catalog number EC90011609, lot number 09C008 purchased in 2014 from Sigma, St. Louis, MO) was authenticated through STR PCR (AmpFLSTR SGM Plus PCR Amplification Kit, Life Technologies) by European Collection of Cell Cultures (ECACC, original source of the cell line, test data 14/04/2009). TPC1 papillary thyroid carcinoma cell line, established by Dr. Nobuo Sato (Cancer Research Institute, Kanazawa University, Japan) and housed and authenticated by MDR, are grown in DMEM with 10% fetal bovine serum and NEAA (non-essential amino acids). All cells were used at passage 1 to 6 for all experiments.

### Generation of stable cell lines

Flag-tagged SDHD WT, SDHD-G12S, and SDHD-H50R plasmids, or shPTEN were transfected into HEK293, Nthy-Ori-3 and TPC1 cells with Lipofectamine<sup>TM</sup> 3000 transfection reagent (Thermo Fisher, Waltham, MA). Fresh medium was replaced 8 h post transfection. Stably transfected cells were selected using G418 (50 µg for HEK293 cells, 300 µg for Nthy-Ori-3 cells) in culture for over 3 weeks. Cell colonies were identified for a positive transfection by Western blot analysis

### Immunoprecipitation for ubiquitination and acetylation

Cell lysates were collected in RIPA buffer and incubated with PTEN antibody (Clone 6H2.1, Cascade Biosciences, Winchester, MA) or FOXO3a antibody (Clone D19A7, Cell Signaling Technology, Danvers, MA) overnight at 4°C followed by protein A/G beads incubation for an additional 2 h. After centrifugation at 800 g for 5 min, the supernatants were removed and cell pellets washed in RIPA buffer, incubated at 4°C for 10 min, then centrifuged again at 800 g for 5 min. Pellets were washed three times and re-suspended in protein loading buffer. Samples were boiled for 5 min and centrifuged at maximum speed for 1 min. Supernatants were collected for Western blot analysis.

### Nuclear-cytoplasmic fractionation

Nuclear-cytoplasmic fractionation was performed as previously described (6). Briefly, cytoplasmic proteins were isolated in buffer A (10 mM HEPES, 1.5 mM Potassium Chloride, 1% Triton X-100, 1 mM Dithiothreitol, protease inhibitor cocktail and phosphatase inhibitor) and nuclear proteins were isolated in Nuclear Extraction Reagent (Pierce, Rockford, IL).

### Western blotting

Whole cell lysates were harvested in M-PER buffer (Pierce, Rockford, IL) with protease and phosphatase inhibitors. Samples were electrophoresed on 4–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes and probed with protein specific primary antibodies followed by incubation with secondary antibody. Target proteins were detected using the Odyssey infrared imaging system (Li-COR, Lincoln, NE). The following primary antibodies were used for detection of specific proteins: FOXO3a, Ubiquitin and LC3B specific antibodies were used at 1:1000 dilution (Cell signaling); P62 specific antibodies were used at 1:500 dilution (Santa Cruz Biotech, Dallas, TX). Best representative blots of results from three separate experiments have been shown in the manuscript for all Western blotting results

### Quantitative-reverse transcription (RT) PCR for detection of FOXO3a target gene expression

Total RNA was extracted from transfected cells using the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol and subsequently treated with DNase I (Invitrogen, Carlsbad, CA). DNase-treated total RNA was reverse-transcribed into cDNA using qScript cDNA SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD) as specified by the manufacturer. Quantitative PCR was performed on a LightCycler 480 (Roche Diagnostics, Basel, Switzerland) using TaqMan Probe ready mix for BECLIN1 and ATG12, and GAPDH as internal control. Primers for BECLIN1 mRNA: forward sequence: 5'-TGGGGAGGTTAGG ATTTGGGA-3', reverse sequence: 5'-GAGCCGTAGGGTGGAAAGC-3'. Primers for ATG12 mRNA: forward sequence: 5'-TAGAGCGAACACGAACCATCC-3', reverse sequence: 5'-CACTGCCAAAACACTCATAGAGA-3'.

### Cyto-ID staining for autophagy

Cyto-ID staining kit (Enzo Life Sciences, Farmingdale, NY) was used to detect autophagy in HEK293, Nthy-Ori-3 and TPC1 cells according to the manufacturer's protocol. Briefly, cells were

plated on cover slips at 70% confluency and allowed to attach overnight. Cell medium was aspirated followed by carefully washing twice using 1x Assay Buffer with 5% FBS. Cells were incubated with Cyto-ID Green Detection Reagent for 30 min at 37°C in the dark. Cells were carefully washed twice with 1X Assay Buffer to remove excess dye and observed immediately under a confocal microscope.

### Statistical analysis

Statistical analysis was performed using Prism for Macintosh (GraphPad Software, La Jolla, CA) using the two-tailed t-test. Data are expressed as means  $\pm$  SEM as indicated. Results were considered statistically significant at a P value of  $< 0.05$ .

### Supplementary Material

Supplementary Material is available at HMG online.

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