FAM129A regulates autophagy in thyroid carcinomas in an oncogene-dependent manner

Bruno Heidi Nozima1, Thais Biude Mendes1, Gustavo José da Silva Pereira2, Rodrigo Pinheiro Araldi1, Edna Sadayo Miazato Iwamura3, Soraya Soubhi Smaili2, Gianna Maria Griz Carvalheira1 and Janete Maria Cerutti1

1Division of Genetics, Department of Morphology and Genetics, Genetic Bases of Thyroid Tumors Laboratory, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil
2Department of Pharmacology, Calcium Signaling and Cell Death Laboratory, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil
3Laboratório de Patologia Molecular, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil

Correspondence should be addressed to J M Cerutti: j.cerutti@unifesp.br

Abstract

We previously proposed that high expression of FAM129A can be used as a thyroid carcinoma biomarker in preoperative diagnostic exams of thyroid nodules. Here, we identify that FAM129A expression is increased under nutrient and growth factor depletion in a normal thyroid cell line (PCCL3), overlapping with increased expression of autophagy-related protein and inhibition of AKT/mTOR/p70S6K. Supplementation of insulin, TSH and serum to the medium was able to reduce the expression of both FAM129A and autophagy-related protein and reestablish the AKT/mTOR/p70S6K axis. To determine the direct role of FAM129A on autophagy, FAM129A was transfected into PCCL3 cells. Its overexpression induced autophagic vesicles formation, evidenced by transmission electron microscopy. Co-expression of FAM129A and mCherry-EGFP-LC3B in PCCL3 showed an increased yellow puncta formation, suggesting that FAM129A induces autophagy. To further confirm its role on autophagy, we knockdown FAM129A in two thyroid carcinoma cell lines (TPC1 and FTC-236). Unexpectedly, FAM129A silencing increased autophagic flux, suggesting that FAM129A inhibits autophagy in these models. We next co-transfected PCCL3 cells with FAM129A and RET/PTC1 and tested autophagy in this context. Co-expression of FAM129A and RET/PTC1 oncogene in PCCL3 cells, inhibited RET/PTC1-induced autophagy. Together, our data suggest that, in normal cells FAM129A induces autophagy in order to maintain cell homeostasis and provide substrates under starvation conditions. Instead, in cancer cells, decreased autophagy may help the cells to overcome cell death. FAM129A regulates autophagy in a cell- and/or context-dependent manner. Our data reinforce the concept that autophagy can be used as a strategy for cancer treatment.

Key Words

- FAM129A
- C1orf24
- thyroid carcinoma
- autophagy
- Niban

Introduction

Thyroid cancer incidence has been increasing over the last three decades worldwide (Howlader et al. 2015, La Vecchia et al. 2015). As thyroid cancer typically presents as a thyroid nodule, proper diagnosis is required for management decision. The gold standard method for the differential diagnosis of a thyroid nodule is the fine-needle aspiration cytology (FNAC). However, nearly 30% of FNACs yield an indeterminate result, which comprises Bethesda III–V categories. Although the risk of malignancy varies from 10 to 75%, depending on the Bethesda classification, most of the patients undergo surgery for a definitive diagnosis (Haugen et al. 2016, Liu et al. 2016).
In an effort to reduce unnecessary surgeries, we previously searched for molecular markers that could help to refine the preoperative diagnosis of indeterminate thyroid nodules. We developed a predictive model, based on the expression of four carcinoma markers (FAM129A, ITM1, ARG2 and DDIT3), combined with the expression of a marker for Hürthle cell adenoma (PVALB). The antibody-based panel significantly improved the ability to distinguish benign from malignant thyroid nodules (Cerutti et al. 2004, 2006, 2011).

Among these markers, FAM129A (aliases C1orf24, NIBAN and GIG39) has been reported to be overexpressed in various human cancer, strengthening its pathogenic relevance (Majima et al. 2000, Adachi et al. 2004, Matsumoto et al. 2006, Ito et al. 2009, Thomas et al. 2016). FAM129A (family with sequence similarity 129 member A) gene is mapped on chromosome 1q25.3 and codifies a protein predominantly expressed in the cytoplasm. The encoded protein may play a role in regulating cell death.

FAM129A was initially identified as differentially expressed in Tsc2 mutant rats, a model of hereditary renal carcinoma (Majima et al. 2000). The absence of expression in normal rat/human kidneys, associated with its overexpression in other renal carcinogenesis model, sporadic human renal carcinomas and renal carcinoma cell lines, supported their hypothesis that FAM129A is a marker of renal carcinogenesis. The authors suggested that FAM129A function as a protector against cell death under stress conditions (Adachi et al. 2004).

Others have showed that FAM129A is induced under endoplasmic reticulum (ER) stress in few cell types such as liver, kidney and cerebrum and may function to link and coordinate ER stress and mTOR/S6K1 pathway (Sun et al. 2007).

Moreover, it was demonstrated that ultraviolet irradiation induced FAM129A phosphorylation by AKT in human glioblastoma and lung cancer cells with subsequent proteasomal degradation of p53, suggesting that FAM129A protected cells from genotoxic stress-induced apoptosis (Ji et al. 2012).

Although it has been suggested that FAM129A is upregulated in response to different stress conditions to protect cells against cell death, its precise role in regulating cell survival and death and the mechanism by which FAM129A acts is not completely understood. Considering that autophagy is rapidly upregulated under cell stress, such as nutrient starvation, growth factor depletion, reactive oxygen species (ROS) accumulation and ER stress (Kroemer et al. 2010, Qin et al. 2010, Zhao & Goldberg 2016), as well as that FAM129A is induced by different stress conditions in different cell models, we here investigated the expression of FAM129A under nutrient starvation and explored its role on autophagy in thyroid carcinoma cell lines.

Materials and methods

Cell culture

The Nthy-ori 3–1 (human thyroid follicular epithelial derived from normal thyroid tissue), WRO and FTC-236 (human follicular thyroid carcinoma cell lines), TPC1 (human papillary thyroid carcinoma cell lines) and PCCL3 (differentiated rat normal thyroid cell line) were grown as described in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Nutrient starvation and growth factor withdrawal

To investigate whether FAM129A expression is induced under stress conditions, both PCCL3 and FTC-236 cells were submitted to nutrient starvation and growth factor depletion, as previously described (Malaguarnera et al. 2014). Briefly, PCCL3 or FTC-236 cells, which require TSH and insulin to growth, were placed in culture medium without TSH for 48 h. The following day, the medium was changed to TSH, insulin and fetal bovine serum (FBS)-deprived medium for 24 h. After 24 h of incubation without TSH, insulin and FBS, the medium was aspirated and the cells were either treated with TSH (1 mU/mL), insulin (10 µg/mL), TSH combined with insulin or FBS for 20 min. After treatment, cells were collected to Western blot analysis. TPC1 cells were subjected to nutrient withdrawal by replacing growth medium for Earle’s Balanced Salt Solution (EBSS – Sigma-Aldrich) for 1 h. After treatment, cells were collected to Western blot analysis.

Western blot analysis

Cells were washed with ice-cold PBS, harvested on ice and lysed with ice-cold lysis buffer (50 mM Tris–HCl, 100 mM NaCl, 50 mM NaF, 1 mM NaVO₄ and 0.5% Triton-X solution) supplemented with the protease inhibitor Cocktail tablets (Roche). Protein concentration was determined by BCA Protein Assay Kit (Pierce, Biotechnologies). About 30 µg of total protein were resolved in 8–12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked for non-specific binding with 5% non-fat dried milk in 1× TBST buffer and incubated at 4°C.
for at least 16 h with the indicated antibodies, as follows: anti-FAM129A (1:200, generated in-house (Cerutti et al. 2004)), anti-LC3B (1:1000, Cell Signaling), anti-p-p70S6K (T389) (1:300, Cell Signaling), anti-p-Akt (S473) (1:1000, Cell Signaling), anti-p-ERK1/2 (T202/Y204) (1:1000, Cell Signaling), anti-V5-Tag (1:2000, Thermo Fisher Scientific), and anti-α-tubulin (1:10,000) (Sigma-Aldrich). Secondary antibodies conjugated with horseradish peroxidase anti-rabbit (1:10,000, Dako) or anti-mouse (1:10,000, Santa Cruz Biotechnology) were used. The membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Merck). The chemiluminescent signal was detected by ImageQuant LAS 4000 system (GE Healthcare). The band intensities were quantified using the ImageJ software (NIH) and corrected for background differences. The data were normalized to the expression of α-tubulin (loading control) and expressed as fold-change.

**Generation of plasmid constructs**

The full-length cDNA of human FAM129A was synthesized from RNA isolated from a follicular carcinoma thyroid sample, which presented high levels of FAM129A expression, using SuperScript III (Life Technologies), according to manufacturer’s recommendations. An aliquot of the cDNA was used as template in a 50 μL PCR reaction. To insert HindIII and BamHI restriction sites at the 5′ and 3′-termini of the PCR product, a sense primer containing HindIII (underlined) and an antisense primer with BamHI (underlined) were used to generate PCR products, as follows: sense: 5′ CCGAAGCTTCAGTTTCCGCGCTCAGCACAGG 3′ and antisense: 5′ CCGGGATCCCTCCTCTGAGGGCAGCAGG TCTGGG 3′. The full-length cDNA of RET/PTC1 was synthesized from a RNA isolated from a RET/PTC1-positive sample (Bastos et al. 2015). An aliquot of the cDNA was used as template to PCR amplification. To insert KpnI sites (underlined) at the 5′ and 3′-termini of the PCR product, a sense 5′ CCGGGTACCGCCGCCGGCATGGCCGACACGC 3′ and antisense 5′ CCGGGTACCAGTTTCCGCGCTCAGCACAGG AATTCT 3′ primers were used to generate PCR products. The amplified products were purified, digested with respective endonucleases and cloned into pBudCE4.1 vector (Invitrogen). Final constructs were sequenced using BigDye Terminator v3.1 kit (Applied Biosystems) and carried out on an ABI3130xl sequencer (Applied Biosystems) equipment.

**PCCL3 cell line transfections**

FAM129A construct or empty vector (pBudCE4.1) were transfected into PCCL3 cell line by electroporation. Briefly, about 5×10⁶ cells were resuspended in electroporation buffer (PBS with 0.1mM CaCl₂, 0.1% glucose) and electroporated with 5 μg of each DNA construct at 150V and 900μF using the Gene Pulser II Electroporation System (Bio-Rad). Pools were selected in complete medium supplemented with 300μg/mL Zeocin (Gibco).

**Monitoring autophagosomes formation by transmission electron microscopy in PCCL3 cells expressing FAM129A**

PCCL3 cells with ectopic expression of FAM129A and control (empty vector) were fixed and stained as previously described (Mendes et al. 2016). Cells were fixed in Karnovsky buffer (4% paraformaldehyde and 2% glutaraldehyde in 0.05M sodium cacodylate buffer, pH 7.2) (Sigma-Aldrich). Post-fixation was performed in 0.1% osmium tetroxide and 0.1M cacodylate buffer for 2h. Then, cells were dehydrated with a graded acetone series, infiltrated in Spurr’s resin (Electron Microscopy Sciences, 14300) overnight and stained with 2% uranyl acetate and lead citrate. Finally, Philips EM 208 transmission electron microscope (FEI Ltd, Eindhoven, The Netherlands) was used to visualize the autophagosomes.

**Plasmid, retroviral production and infection in PCCL3 expressing FAM129A**

Viral preparation was performed as previously described (Pereira et al. 2017). Briefly, about 15μg of mCherry-eGFP-LC3B construct and 5μg of the vesicular stomatitis virus G protein vector were co-transfected into the 293 gp/bsr cell line by electroporation. Retroviral particles in the supernatant were supplemented with polybrene (4μg/mL), after 48h of transfection and stored at −80°C. PCCL3 cells (5×10⁴) with stable expression of FAM129A or control (empty vector) were seeded in 35 mm dishes and, the next day, infected with supernatant containing retroviral particles for 24h. The plasmid encoding mCherry-eGFP-LC3B construct was kindly provided by Dr Mauro Piacentini (University of Rome Tor Vergata, Rome, Italy).

**Autophagic flux by confocal microscopy in PCCL3 cells expressing FAM129A**

PCCL3 cells with overexpression of FAM129A, infected with mCherry-eGFP-LC3B vector, were seeded in coverslips, fixed in 4% paraformaldehyde after 48h, washed in PBS and mounted in Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Fluorescent signal
was detected in Zeiss LSM 780 Confocal Microscopy (Carl Zeiss), equipped with EC Plan-Neofluar Objective Lens (40×/0.7 PH2) and AxioCam HRC (Carl Zeiss). Wave lengths filters of 488 and 595 nm were used to detect green and red signals, respectively. Puncta formation was determined in ImageJ software using Watershed Segmentation plug-in. Twenty-five random cells were analyzed for yellow and red puncta formation, indicating autophagosome and autolysosome formation, respectively.

**Autophagic flux in thyroid cancer cells**

To investigate whether FAM129A interferes with autophagic flux in thyroid cancer cell lines (TPC1 or FTC-236), the cells (3 × 10⁴) were seeded in 35 mm dishes for retroviral infection, as described above. Subsequently, the cells were infected with supernatant containing retroviral particles and submitted to selection with puromycin for 48 h. Finally, cells expressing mCherry-eGFP-LC3B vector were transfected with pre-designed validated siRNA against the human FAM129A (siRNA ID 128925) or transfected with scramble siRNA (siRNA ID 4390846) (Ambion Inc.) as previously described (Carvalheira et al. 2015). Briefly, 100 nM of pre-designed siRNAs were transiently transfected into 2.5 × 10⁶ cells in electroporation buffer, at 150 V and 900 µF, using the Gene Pulser II Electroporation System (Bio-Rad). Autophagic flux was determined by confocal microscopy, as described above. To monitor the effect of FAM129A knockdown on signaling pathways, cell lysates from silenced and control cells were obtained 72 h post transfection and analyzed by Western blot.

**Co-expression of FAM129A and RET/PTC1 in PCCL3 cells**

To better understand the role of FAM129A in regulating autophagy, we co-expressed FAM129A and RET/PTC1 in PCCL3 cells. Five micrograms of each DNA construct were transiently transfected alone or in combination as described above. Total protein was isolated 72 h of transfection and analyzed by Western blot. The band intensities were quantified using the ImageJ software (NIH) and corrected for background differences. The data were normalized to the expression α-tubulin (loading control) and to the expression of V5 tag, which indicates RET/PTC1 expression.

**Statistics**

Quantitative data were submitted to Shapiro–Wilk Normality Test and Levene’s Test to assess the equality of variances. The data that met the parametric assumptions were submitted to Student’s t test or one-way ANOVA. As nonparametric test, Mann–Whitney or Kruskal–Wallis (followed by Dunn’s posttest) were used. P<0.05 were considered statistically significant. Tests were performed in Stata12 (StataCorp LLC) and GraphPad Prism (GraphPad Software).

**Results**

**Expression of FAM129A in thyroid cell lines**

To define whether FAM129A is induced under stress conditions and its direct effects on expression of autophagy markers, we initially tested the endogen expression of FAM129A in two normal follicular thyroid (Nthy-orí 3–1 and PCCL3) and three thyroid carcinoma cell lines (WRO, FTC-236 and TPC1) by Western blot analysis. As both the normal thyroid rat cell line (PCCL3) and the thyroid carcinoma cell line (FTC-236) are maintained in medium containing TSH and insulin, they were selected to test the effects of nutrient deprivation and growth factors withdrawal (TSH, Insulin) in the expression of both FAM129A and autophagy-related protein.

The TPC1 and FTC-236 cell lines were used to investigate the effects of FAM129A knockdown on autophagy (Supplementary Fig. 1).

**Metabolic stress-induced FAM129A and autophagy-related protein in PCCL3 cell line**

To determine whether FAM129A expression is induced under nutrient starvation or growth factor depletion, PCCL3 cell line were maintained in medium without TSH, insulin and serum. FAM129A expression was induced at very high levels in PCCL3 cells maintained in reduced medium, compared to the cells in complete medium (P<0.01) (Fig. 1A). Notably, in the normal thyroid rat cell line, a small size of FAM129A protein (70 kDa) was upregulated under stress conditions. This smaller band was observed only in rat cell line, which is similar to data reported previously (Adachi et al. 2004).

Upon a 72 h of growth factors depletion, followed by replacement of insulin and TSH (alone or in combination) or FBS for 20 min, FAM129A expression was reduced (Fig. 1A). Interestingly, FAM129A expression was restored, at basal levels, when FBS was re-introduced (P<0.01, Fig. 1A).

As autophagy process is activated as an adaptive response to different forms of metabolic stress, we next...
assessed the level of LC3-I (16 kDa) and LC3-II (14 kDa) protein expression, which is considered to be one of the most reliable markers of the autophagy process. As the ratio of LC3-II/α-tubulin is considered an accurate index of autophagy, relative levels of LC3-II protein were assessed. Although basal level of autophagy was detected in PCCL3 cells, higher ratio of LC3-II/α-tubulin was observed in PCCL3 cells under metabolic stress (Fig. 1A). The ratio of LC3-II/α-tubulin was restored to baseline ($P<0.05$) when insulin (alone or in combination with TSH) were added to the medium. Even though FAM129A expression was restored when serum was replaced, the level of LC3-II was not reestablished to baseline levels when serum was replenished. These data suggest that the effect of serum replacement on expression of LC3-II in PCCL3 cells might be time dependent.

Altogether, these data indicated that elevated expression of FAM129A, under stress condition, can trigger autophagy.

Metabolic stress inhibited AKT/mTOR/p70S6K and activated ERK1/2 signaling pathways in PCCL3 cell line

As autophagy integrates multiple stress-sensitive signaling pathways, we investigated key signaling pathways that have been associated with autophagic process in response to nutrient and growth factors depletion such as AKT/mTOR/p70S6K and ERK1/2 signaling pathways. Nutrient and growth factors depletion inhibited AKT/p70S6K signaling pathway, while activated the ERK1/2 signaling pathway (Fig. 1B).

Insulin (alone or in combination) or serum restored the phosphorylation levels of AKT (S473), while the treatment with TSH alone did not decrease AKT phosphorylation. Upon treatment with insulin, TSH and serum, the p70S6K phosphorylation at T389 was restored at baseline levels, while the treatment with insulin and TSH (in combination) increased the p70S6K phosphorylation at T389. These data suggest that TSH-induced phosphorylation of mTOR substrate p70S6K may not require AKT. ERK phosphorylation at T202 and T204 was partially inhibited by stimulation with TSH alone. When cells were stimulated with insulin (alone or in combination) or the serum was replaced into the medium, ERK phosphorylation was further reduced (Fig. 1B).

In summary, these data suggest that metabolic stress, which we found associated with increased expression of FAM129A and autophagy-related proteins in PCCL3 cells, inhibited AKT/mTOR/p70S6K while activated the...
ERK signaling pathway. Whether another pathway is involved in TSH-induced p70S6K activation and thus modulates autophagy, it is not clear.

**FAM129A overexpression induces autophagy in PCCL3 cell line**

In order to validate the direct effect of FAM129A expression on autophagy, the cDNA of FAM129A was cloned in pBudCE4.1 expression vector, under CMV promoter and transfected into PCCL3 cells. Autophagic process was monitored in FAM129A overexpressing cells and control using transmission electron microscopy (TEM). Although few autophagic vesicles were found by TEM within control cells, they were abundant through the cytoplasm within cytoplasm of FAM129A-expressing cells (Fig. 2A). These results suggested a direct effect of FAM129A on autophagy.

To confirm these results, autophagic flux was determined by transfecting FAM129A expressing cells and control with a mCherry-eGFP-LC3B vector. The double-tagged LC3B strategy, to distinguish autophagosomes from autolysosomes, has been largely used to monitor autophagic flux (Klionsky et al. 2016). When autophagy is induced, LC3B is lapidated to autophagosome membrane creating yellow puncta in the cytoplasm, as a result of mCherry and eGFP overlapping signals. As autophagy progresses, autophagosomes fuse with lysosomes generating autolysosomes and eGFP is degraded resulting in red puncta signal in the cytoplasm. We showed that FAM129A overexpression increased yellow puncta formation, suggesting that FAM129A induces autophagosome formation (Fig. 2B).

These results provided further evidence that FAM129A overexpression in associated with autophagy in PCCL3 cells.

**FAM129A has opposed effect on autophagy in cancer cells**

As FAM129A induced autophagy in normal thyroid cells, we next used siRNA strategy to knockdown FAM129A and confirm whether its silencing would reduce autophagy rate. The papillary thyroid carcinoma cell line (TPC1) and the follicular thyroid carcinoma cell line (FTC-236) were used as models.

The TPC1 and FTC-236 cells transfected with mCherry-eGFP-LC3B and subsequently transfected with...
FAM129A-specific siRNA were imaged and red and yellow puncta quantified (Fig. 3). The FAM129A knockdown efficiency in TPC1 and FTC-236 cells was confirmed by Western blot analysis (Fig. 4 and Supplementary Fig. 2). FAM129A silencing in both cancer cell lines resulted in a marked increase number of yellow (autophagosome) and red puncta (autolysosome) when compared to scramble siRNA (control group) (Fig. 3). These findings suggested that FAM129A inhibits autophagy in both cancer cell lines.

As FAM129A showed inhibitory effect on autophagy in thyroid cancer cells, we transfected TPC1 cells with siRNA to knockdown FAM129A and cultured the cells in nutrient depleted medium. When TPC1 cells were transfected with scramble siRNA (control) and autophagy was induced by amino acid starvation, FAM129A expression was induced (Fig. 4A). Additionally, FAM129A knockdown slightly increased the LC3-II expression. A much higher effect on LC3-II expression was observed when TPC1 transfected with siRNA to knockdown FAM129A were culture under stress conditions. These findings confirmed that FAM129A inhibited autophagy in TPC1 cancer cells.

Nutrient-sensitive pathways were also modulated by FAM129A silencing. Upon starvation, the phosphorylation of the mTOR substrate p70S6K was reduced in TPC1. Interestingly, FAM129A knockdown in TPC1 cells reduced the p70S6K (T389) and AKT (S473) phosphorylation, suggesting that both FAM129A and metabolic stress regulates phosphorylation of p70S6K and AKT. Metabolic stress in TPC1 cells increased ERK phosphorylation at T202 and T204, no additional effect was observed following FAM129A silencing (Fig. 4B).

Once FTC-236 cell line requires TSH and insulin to grow, cells were cultured in serum and growth factor-free medium. Under nutrient and growth factor depletion, FAM129A expression was significantly increased. When medium was replenished and insulin or serum was added to the media, FAM129A expression was decreased. However, FAM129A expression level was significantly reduced when cells were culture in insulin and TSH-supplemented medium, which was accompanied by higher expression of LC3-II (Fig. 5A), corroborating the data that FAM129A knockdown in FTC-236 cells induced autophagy.

However, phosphorylation of p70S6K and AKT was not affected by metabolic stress or even when cells were maintained in medium supplemented with insulin, TSH (alone or combination) or serum (Fig. 5B).
Altogether these data suggest that FAM129A may modulate autophagy in a PI3K/AKT-dependent and -independent manner.

**FAM129A dual effect on autophagy is context dependent**

As FAM129A induced autophagy in PCCL3 cells, while its expression in thyroid carcinoma cell lines was associated with inhibition of autophagic process, we postulated that the effect of FAM129A on autophagy of thyroid cells is context dependent.

As TPC1 cell line is a papillary-derived cell line with RET/PTC1 fusion as a driver, we next transiently transfected RET/PTC1- and FAM129A-expressing vectors into PCCL3 (alone or in combination) and tested whether RET/PTC1 would interfere with autophagic process previously observed in PCCL3 expressing FAM129A.

As expected, the LC3-II expression was significantly higher in PCCL3 expressing FAM129A than that observed in control cells. Interestingly, ectopic expression of RET/PTC1 further increased LC3-II expression, while the expression of LC3-II was significantly lower in PCCL3 cells co-transfected with FAM129A and RET/PTC1, compared to controls (Fig. 6).

These results indicated that FAM129A might inhibit autophagy in a RET/PTC1-driven tumor (Fig. 6).

**Discussion**

In this study, we observed that in normal thyroid rat cell line PCCL3, nutrient and growth factors depletion induced both FAM129A and autophagy-related protein, while inhibits AKT/mTOR/p70S6K signaling pathway. The baseline autophagy was increased to supply limited nutrients and sustain cell survival. In response to nutrient and growth factor availability, FAM129A expression reduced while the AKT/p70S6K axis was activated leading to suppression of autophagy. Notably, the phosphorylation of ERK was significantly increased by nutrient and growth factor withdrawal. Its phosphorylation level was significantly reduced when cells were cultured in insulin, TSH and serum-supplemented medium.

Although it is not clear whether there is a crosstalk between PI3K/AKT/mTOR/p70S6K and MAPK/ERK pathway to regulate autophagy, it has been suggested that RAS protein might be involved in the intersection of MAPK/ERK and PI3K/AKT signaling pathways. RAS stimulates autophagy when the MAPK signaling pathway is activated, while inhibits autophagy when
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PI3K/AKT is activated (Furuta et al. 2004, Morani et al. 2014, Netea-Maier et al. 2015, Weckman et al. 2015). Whether an alternative pathway, involved in the TSH-induced activation of p70S6K in normal cells, may play a role in autophagy induction in thyroid cells, need further investigation.

To more directly investigate the role of FAM129A on autophagy, both the presence of autophagic vesicles and the autophagic flux were evaluated in PCCL3 cells. Autophagic events, such as autophagosomes and autolysosome, became detectable in normal thyroid cells with stable expression of FAM129A.

We further assessed the effects of FAM129A on autophagy in thyroid carcinoma cell lines TPC1 and FTC-236. Surprisingly, FAM129A knockdown inhibited autophagy and reduced the phosphorylation of AKT and p70S6K in TPC1 cells, suggesting that FAM129A induces autophagy in these cell lines through AKT/mTOR/p70S6K signaling pathway.

In FTC-236 cells, nutrient and growth factor withdrawal increased FAM129A expression. Although FAM129 expression was reduced and the expression of autophagy-related protein increased in insulin and TSH-supplemented media, the phosphorylation of AKT/p70S6K and ERK did not change. That is not surprising, as FTC-236 is a PTEN-null and TP53-mutated cell line, and it has been demonstrated that PTEN is necessary to suppress phosphorylation of AKT (He et al. 2012) and that P53 regulates autophagy via mTOR-dependent and independent pathway (Qu et al. 2003).

In summary, these data suggest that FAM129A likely inhibits autophagy via both AKT/mTOR/p70S6K-dependent and -independent pathway in thyroid cancer cells.

Based on FAM129A ability to induce autophagy in normal thyroid cell line and to inhibit autophagy in thyroid carcinoma cell lines, we hypothesized that FAM129A may play a dual role in the control of autophagy in thyroid cells and should be interpreted in these contexts.

As TPC1 cell lines harbor the RET/PTC1 oncogene, we co-expressed RET/PTC1 and FAM129A transcripts in PCCL3 normal thyroid cells and investigated the expression of autophagy-related protein. When RET/PTC1 and FAM129A were co-expressed in PCCL3 cells, LC3-II expression was significantly lower than the expression of RET/PTC1 alone. These findings suggested that, RET/PTC1 upregulated the baseline autophagy in PCCL3 cells, which was significantly repressed by FAM129A expression,

Figure 5
Western blot analysis of Follicular Thyroid Carcinoma FTC-236 cells cultured under growth factors depleted medium and following insulin and TSH (alone or combination) or FBS replacement. (A) Expression of FAM129A and LC3-I/II. (B) Activity of main regulators of autophagy AKT/mTOR and MAPK/ERK, measured by p-p70S6K (T389), p-AKT (S473) and p-ERK (T202/T204). The graphics show the densitometry analysis of each protein normalized by α-Tubulin. Data expressed as mean ± s.d. All experiments were performed in triplicate. *P < 0.05, **P < 0.01 and ***P < 0.001.
suggesting that FAM129A inhibited autophagy induced by RET/PTC1 oncogene.

Similar to what we observed when RET/PTC1 oncogene was transfected into PCCL3 cells, others have also demonstrated that BRAF V600E and RAS mutations, which also constitutively activates MAPK/ERK pathway, upregulated autophagy in thyroid cancer cells (Morani et al. 2014).

These findings called into question why BRAF, RAS and RET/PTC1 transformed cells increase basal autophagy rate, together with the expression of FAM129A, which will ultimately reduce the expression of autophagy-related protein.

Oncogene-induced senescence (OIS) is a well-established event that occurs very early during tumorigenesis and is a critical step in tumor suppression. It was described in primary human thyrocyte transfected with BRAF V600E, HRAS and RET/PTC1 oncogenes and associated with growth arrest triggered by the enforced expression of cancer driver genes (Vizioli et al. 2011). An elegant study verified that autophagy was activated upon acute induction of OIS, in order to contribute to tumor suppression by controlling the senescence phenotype (Narita et al. 2009). Therefore, it would work as a pro-senescence mechanism. If tumor cells somehow bypass senescence, then autophagy might help the cells to survive under metabolic stress. It has been suggested that the level of autophagy might mediate diverse cellular responses.

We have previously shown that FAM129A knockdown induced cell cycle arrest (G0/G1), increased apoptosis and inhibited cell migration in the TPC1 cell line, suggesting that FAM129A increased cell survival via inhibition of apoptosis and induced cell migration (Carvalheira et al. 2015). We here showed that FAM129A knockdown, under stress conditions, inhibited autophagy in TPC1 cell line. Additionally, FAM129A inhibited autophagy in PCCL3 cells transfected with the oncogene RET/PTC1. Therefore, FAM129A might impact cell survival based on its ability to inhibit both apoptosis and autophagy.

Although FAM129A seems to play a role in autophagy, it is still unclear whether FAM129A is a candidate gene that sense lack of nutrients and/or growth factors in thyroid cells or is a downstream effector of signaling pathways that senses lack of nutrients and growth factors such as PI3K/AKT/mTOR and MAPK/ERK and promotes or inhibits depending on the cell context.

Importantly, the loss-of-function mutations in TSC1 and TSC2, key negative regulators of mTOR, lead to renal carcinogenesis in the hereditary renal cancer model. It is acknowledged that loss of TSC2 can result in abnormal activation of PI3K/AKT/mTOR pathway and ultimately autophagy inhibition (Uno et al. 2008). FAM129A was initially identified as expressed in renal tumors developed in Tsc2-mutant rats (Eker) model (Majima et al. 2000). Later, the authors showed that FAM129A is strongly upregulated in the early stages of renal carcinogenesis and may function as a protector against cell death under stress conditions (Adachi et al. 2004).

Additionally, this group demonstrated that FAM129A is phosphorylated by AKT in response to genotoxic (ultraviolet irradiation) stress in human glioblastoma cells. In this cell tumor model, once phosphorylated at Ser602, FAM129A binds to nucleophosmin (NPM), thereby preventing NPM to bind to MDM2 oncoprotein, promoting P53 degradation and increasing cell survival (Ji et al. 2012).

Recently, FAM129A was identified as a novel target of ATF4, a key regulator of the adaptive integrated stress response (ISR). The authors showed that the level of FAM129A can confer a pro-survival function during ISR, through attenuation of the p53-dependent apoptotic responses (Evstaieva et al. 2018).

Several groups have showed that FAM129A is overexpressed in several cancer subtypes (Matsumoto et al. 2006, Ito et al. 2009, Thomas et al. 2016). Additionally, the human protein atlas analysis, which evaluated tissue array based on immunohistochemistry for numerous proteins in normal tissues and over 200 tumor subtypes from 20 different cancer and cell lines, demonstrated that FAM129A is overexpressed in colorectal cancer, breast
cancer, prostate cancer, lung cancer, melanoma and other tumors, including thyroid (Uhlén et al. 2015). Although FAM129A is overexpressed in several tumors and might be overexpressed under different stress conditions, it might favor one pathway over another in a cell-, stress- and mutation type-dependent manner.

These findings suggest that, at least for some tumor types, pharmacological induction of autophagy might be advantageous in the early stages of tumorigenesis (Morani et al. 2014, Netea-Maier et al. 2015).

In conclusion, our results indicated that FAM129A may play a dual role in the regulation of autophagy in thyroid cells. It can increase baseline autophagy in normal thyroid cells in response to nutrient and growth factors depletion or can inhibit autophagy in thyroid carcinomas in the presence of activating mutations in oncogenes, such as RET/PTC1 fusion. It is still not clear whether, after passing the oncogene-induced replicative stress, autophagy might be induced to promote cell survival during tumor progression. Although our data suggest that FAM129A may regulate autophagy in thyroid cells through AKT/mTOR axis, it may also regulate autophagy through additional pathways. An improved understanding of this complex network will contribute to understand the role of FAM129A and autophagy in thyroid tumor progression. Finally, autophagy might be an alternative mechanism to treat different tumors.

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