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Hypoxia-inducible factors, mTOR, and astrin constitute an integrative regulatory network in colon cancer cells

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ABSTRACT

Astrin/SPAG5 is a mitotic spindle protein found to be overexpressed in several human cancers, functioning as an oncogene. The expression of Astrin has not been reported so far in colon cancer, nor has it been related to HIFs expression or action. Since mTOR, Astrin, and hypoxia-inducible factors (HIFs) are involved in promoting the growth and survival of cancer cells, we investigated the possible interaction between them in cultured colon cancer cells. Both Astrin and HIF-1α and HIF-2α protein levels were found only expressed in colon cancer cells compared with nonmalignant cells. Our data indicate that mTOR stimulates both Astrin and HIFs expression, but notably, mTORC activity seems to be independent of Astrin expression levels. However, while HIF-1α or HIF-2α stable knockdown increased Astrin expression, mTOR activity was affected in an opposite way by HIF-1α or HIF-2α silencing, indicating that HIF-1α inhibits mTOR while HIF-2α stimulates its activity. These data suggest that mTOR, Astrin, and HIFs compose an integrative network interacting to activate positive or negative regulatory loops probably to coordinate cancer cell growth, metabolism, and survival under oncogenic stress.

1. Introduction

The Ser/Thr kinase mTOR is a sensor of nutrients, growth factors, and stress signals, acting as a master regulator of growth, metabolism, autophagy, and cell survival in both nonmalignant and cancer cells [1]. Depending on its subunit composition, mTOR exists as two distinct complexes, mTORC1 and mTORC2, both including mTOR and sharing some components. The regulatory-associated protein of mTOR (Raptor) and the negative-regulator proline-rich Akt substrate 40 kDa (PRAS40) is only present in mTORC1. In contrast, the unique rapamycin-insensitive mTOR companion (Rictor) protein and the mammalian stress-activated MAPK interacting protein 1 (mSin1) are only present in mTORC2 [1,2].

The dysregulation of the PI3K/Akt or the Ras/ERK signaling pathways is widespread in many cancer types, including colon cancer, converging both pathways in mTOR activation. In nonmalignant cells, in the presence of nutrients and energy, mTOR induces anabolic processes but, in their absence, inhibits them and activates autophagy. Notably, mTOR inhibits Akt through negative feedback regulation, and thus its hyperactivation would ultimately provoke apoptosis cell death [1–3]. In cancer cells, mTOR becomes activated due to the altered oncogenic signaling pathways and oxidative stress [1]. Cancer cells have evolved protective tools to avoid the mTOR hyperactivation-induced cell death while maintaining mTORC activity and the survival mechanisms it induces. Among them are metabolic reprogramming, induction of antioxidant protein synthesis, and induction of stress granule formation [4]. The Sperm-associated Antigen 5 (SPAG5), or Astrin, is a mitotic spindle protein overexpressed in several human cancers that functions as an oncogene [5,6]. It has been reported that Astrin competes with mTOR for Raptor binding and recruits mTOR to stress granules, suggesting that in this way, Astrin may prevent mTORC1 hyperactivation-induced apoptosis [7]. Stress granules then would function as sentinels of mTORC1 signaling, allowing cancer cells to escape stress-induced cell death [4,7].

In addition to oncogenic mTORC signaling, hypoxia resulting in nutrient depletion is a hallmark of the tumoral microenvironment. The hypoxia-inducible factors (HIFs) regulate the cellular adaptation to oxygen deprivation and play key roles in cancer biology [8,9]. Remarkably, in cancer cells, HIFs upregulation often occurs without hypoxic conditions because its expression can be induced by oncogenic signaling by several mechanisms [10]. The expression of Astrin has not been reported so far in colon cancer, nor has it been related to HIFs expression

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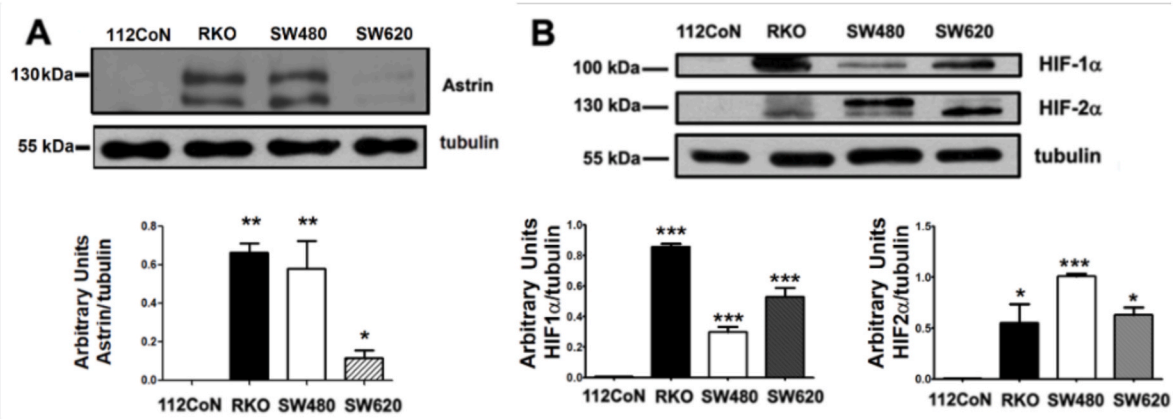

Fig. 1. Astrin and HIFs are expressed only in malignant cells under normoxic conditions. Cell extracts were obtained from non-malignant 112CoN or the indicated colon malignant cells. Samples (50 μg) were separated by 10% SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and analyzed by immunoblotting using antibodies against Astrin (panel A) or against HIFs isoforms (panel B) as indicated in the figure. β-tubulin was used as a control for equal loading. Densitometric analysis was performed to quantify the change in Astrin or HIFs expression levels in cancer cells compared with 112CoN non-malignant colon cells. The bar graphs represent the means ± standard error of the mean (SEM) from at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

or action. In this work, we found that colon cancer cells overexpress Astrin. Our data indicate that mTORC controls both Astrin and HIFs expression, but its activity seems to be independent of Astrin expression levels. However, mTORC activity is affected in an opposite way by HIF-1α or HIF-2α. Our data suggest that mTORC, Astrin, and HIFs constitute an integrative regulatory network in colon cancer cells.

2. Materials and methods

Reagents and antibodies. Hydroxychloroquine and Temsirolimus (CCI-779) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used in the experiments were obtained from the following sources: anti- Astrin/SPAG5 was obtained from Santa Cruz Biotechnology Inc (Sta. Cruz, CA, USA); anti-HIF-1α, anti-HIF-2α, anti-β-tubulin, and anti-Phospho-S6 Ribosomal protein (Ser235/236) were obtained from Cell Signaling Technology (Danvers, MA, USA). Goat anti-mouse and anti-rabbit IgG-horseradish peroxidase-conjugates were from Pierce (Rockford, IL, USA).

Plasmids. The control plasmid containing a scrambled shRNA sequence was obtained from Santa Cruz Biotechnology. The control plasmid (void pSuper), HIF-1α, and HIF-2α RNAi plasmids were donated by Dr. Daniel Chung, and their construction and effectiveness were previously described [11–13].

Cell lines and treatments. All colon cancer cell lines (RKO, SW480, and SW620) and the nonmalignant 112CoN cell line used were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and were authenticated by Short Tandem Repeat DNA profiling in June 2017 at the Instituto Nacional de Medicina Genómica (INMEGEN) in Mexico City. All cells were cultured in a humidified 5% CO_2 incubator at 37 °C. The human RKO and primary SW480 cell lines used in our study have oncogenic driver mutations that result in mTORC1 activation [14].

The inhibition of mTORC1 was determined by incubating cells with the rapamycin analog CCI-779 (Temsirolimus). HIFs-induced autophagy may be abolished to increase the cytotoxicity of mTOR inhibition through hydroxychloroquine (HCQ), which prevents the fusion of autophagosomes with lysosomes, the final step of autophagy [15,16]. The doses used for the experiments performed were the following: 20 μM of HCQ, which had no inhibitory effect on cell viability and was reported as a dose necessary to inhibit autophagy without affecting cell viability [15], and 20 μM of CCI-779 to inhibit phosphorylation of p70 ribosomal S6 Kinase, a typical substrate of mTORC1 [15,16].

Western Blotting. Protein samples (50 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked overnight at 4 °C with 5% nonfat dry milk and then incubated with the indicated primary antibody. Detection was performed using the SuperSignal Kit (Pierce) with a horseradish peroxidase-conjugated second antibody. A β-tubulin antibody was used as the control for equal loading.

HIF-1α or HIF-2α Knockdown. Stable silencing of HIF-1α or HIF-2α was generated as described in Ref. [17]. Briefly, cells were transfected with either 1 μg of the control plasmid or 1 μg of pSuper HIF-1α-RNAi or HIF-2α-RNAi plasmids, which were constructed and donated by Dr. Daniel Chung [11–13]. Stable transfectants were selected with 3 μg/mL puromycin (Sigma) or 5 μg/mL G418 (Sigma), respectively, for four weeks, and the clones were picked and screened for HIF-1α or HIF-2α -silencing by flow cytometry and immunoblot analysis as previously reported [17]. The nucleotide sequences derived from human HIF-1α or HIF-2α mRNA used for generating the knockdowns are described in Refs. [12,13].

Statistical Analysis. The data are expressed as the mean ± standard error of the mean (SEM). Statistical data analysis was performed using Student's t-test or a one-way ANOVA with Tukey's or Bonferroni's comparison tests and was performed with the GraphPad Prism program. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

The mitotic spindle protein Astrin/SPAG5, overexpressed in malignant cells, has been implicated in neoplastic growth, chemoresistance, metastasis, and overall survival of several cancer types [5,6]. HIFs over-expression has been previously reported in many cancer types under normoxic conditions [9], and we have also previously reported this in colon cancer cells [15,17]. To investigate if Astrin is expressed in colon cancer cells, we analyzed its protein levels by Western blotting in several cultured colon cancer cells compared with the levels found in colon nonmalignant cells. Fig. 1 panel A shows that Astrin is expressed as two isoforms only in colon malignant cells (RKO, SW480, and SW620) but not in nonmalignant 112-CoN cells. We also confirmed that both HIF-1α and HIF-2α are also only expressed in colon malignant cells under normoxic conditions (Fig. 1 panel B).

Hypoxia and consequent nutrient depletion, along with a hyperactive metabolism, and oncogenic mTOR signaling, are conditions frequently found in tumors [18]. mTOR coordinates cell growth and metabolism by integrating growth factor signaling with survival signaling in cancer cells, thus functioning as a nutrient and as a stress sensor [1,18]. To investigate the effect of mTORC1 inhibition on HIF-1α or HIF-2α expression, SW480 cells were incubated for 48 h in the

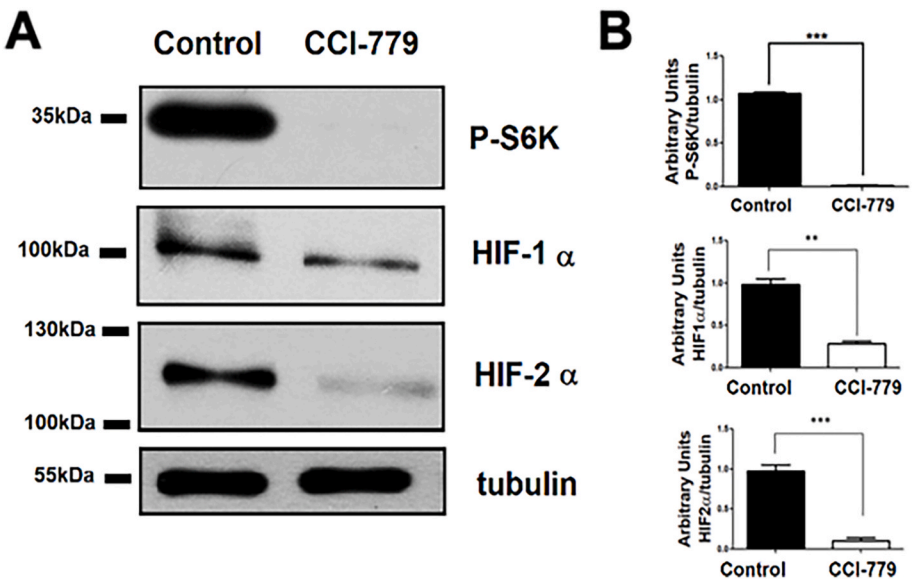

Fig. 2. Inhibition of mTORC with CCI-779 diminishes both HIF-1α and HIF-2α expression levels. SW480 cells were treated with 20 μM CCI-779 for 48 h. A) HIF-1α, HIF-2α and Phospho-S6K expression levels were analyzed in cell extracts by Western blotting. β-tubulin was used as a control for equal loading. B) Densitometric analysis was performed to estimate the changes in PhosphoS6K and HIFs expression levels with respect to β-tubulin expression. The data are presented as the mean values ± SEM from at least three independent experiments, **p < 0.05, ***p < 0.005.

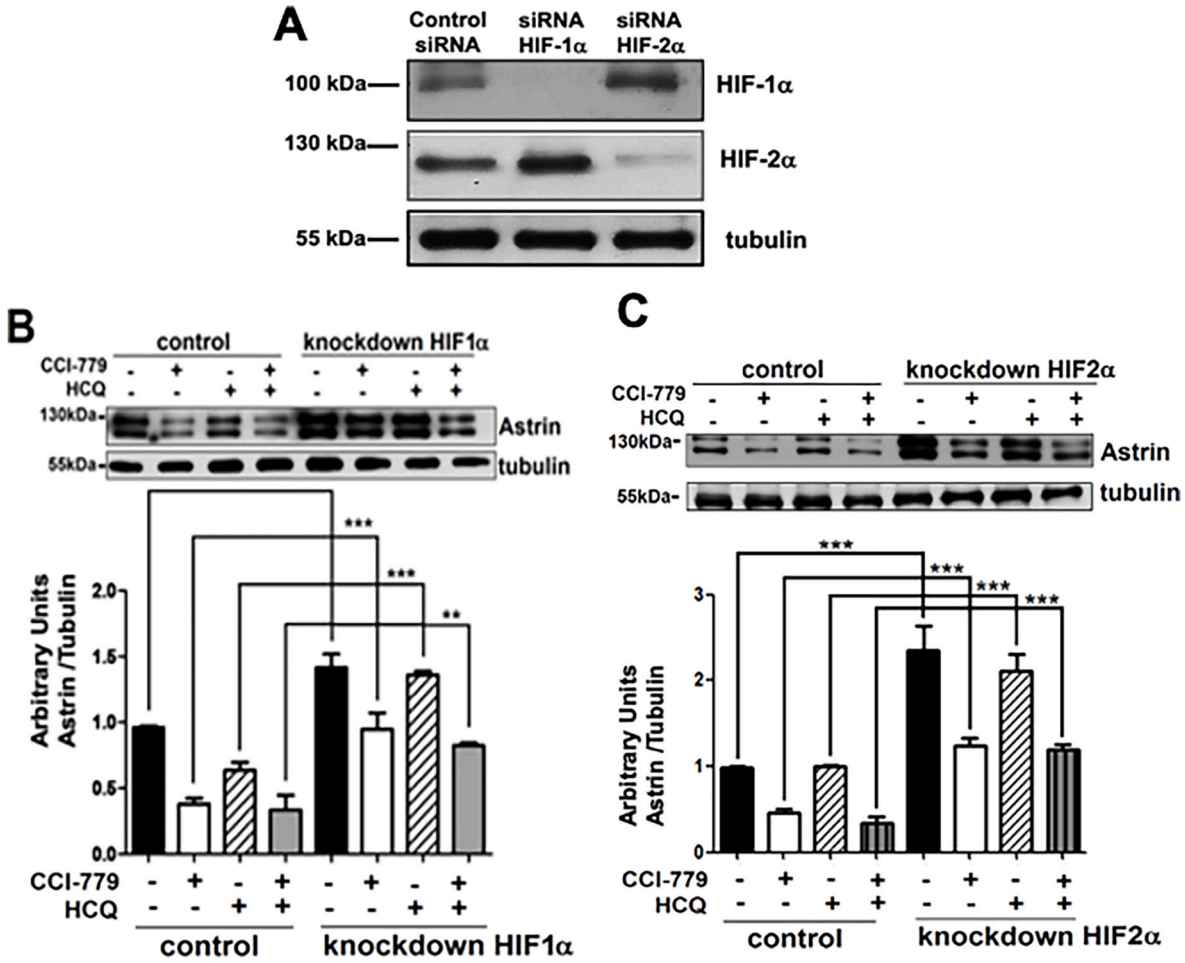

Fig. 3. Knockdown of HIF-1α or HIF-2α increased Astrin expression but the effect of each HIF knockdown on mTORC activity was opposed. A) The efficiency of HIF-1α or HIF-2α knockdown was evaluated in SW480 cells by Western blot. Tubulin was used as a control for equal loading. B–C) Control or HIF-1α (panel B) or HIF-2α (panel C) -silenced SW480 cells were incubated with 20 μM CCI-779 alone, with 20 μM HCQ, or in combination with both agents for 48 h. Astrin expression levels were analyzed in cell extracts by Western blotting. Densitometric analysis was performed in each panel to estimate the Astrin expression with respect to the control β-tubulin expression levels. The data are presented as the mean values ± SEM from at least three independent experiments. **p < 0.01. ***p < 0.001.

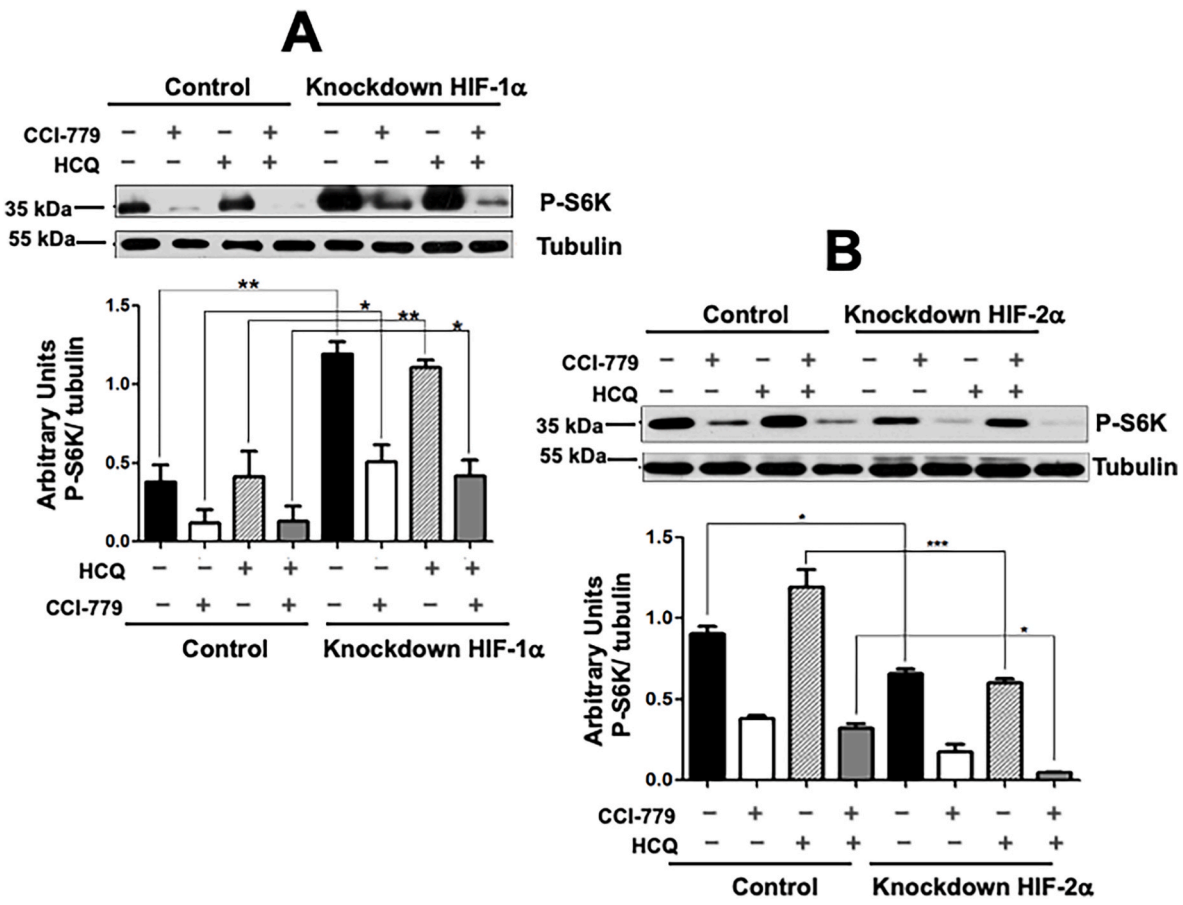

Fig. 4. Effect of Knockdown of either HIF-1α (panel A) or HIF-2α (panel B) in mTORC activity. Control or HIF-silenced SW480 cells were treated with 20 μM CCI-779 alone, with 20 μM HCQ, or in combination with both agents for 48 h. The phosphorylation status of the ribosomal protein S6 was detected as a measure of mTORC activity by Western blotting. β-tubulin was used as a control for equal loading. Densitometric analysis was performed in each panel to estimate the changes in Phosporylated S6K with respect to the control β-tubulin. The results shown in both panels present the mean values ± SEM representative of at least three independent experiments using different cell preparations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

absence or presence of the mTORC1 inhibitor Temsirolimus (CCI-779). The efficiency of mTOR inhibition was demonstrated by the negative effect obtained in the phosphorylation status of the typical mTORC substrate, the p70 ribosomal S6 protein Kinase (p70-S6K, at Thr389), as can be observed in Fig. 2. Consistent with previous reports suggesting that the expression of both HIF-1α and HIF-2α is dependent on mTOR [18–20], the results shown in this figure also indicate that the expression levels of both HIF-1α and HIF-2α obtained by Western blotting and densitometric analysis were significantly diminished by mTORC inhibition.

To block the expression of HIFs, stable transfected SW480 cells with the control scrambled shRNA plasmid, with HIF-1α RNAi, or with HIF-2α RNAi were selected with 3 μg/mL puromycin (Sigma) or 5 μg/mL G418 (Sigma), respectively, during four weeks. The clones were selected, and the silencing efficiency was examined by Western blotting, as shown in Fig. 3A. The stable knockdown of either HIF-1α (Fig. 3 panel B) or HIF-2α (Fig. 3 panel C) in colon cancer cells induced a significant increase in both Astrin isoforms expression levels. In addition, in both panels B and C of Fig. 3, it can also be observed that the inhibition of mTORC1 with the rapamycin analog CCI-779 diminished Astrin expression, alone or in combination with the autophagy flux inhibitor Hydroxychloroquine (HCQ), which alone did not produce any significant effect. Thus, combining the results obtained in Figs. 2 and 3, data indicate that mTORC inhibition negatively affects both Astrin and HIFs expression.

However, when we investigated the effect of HIFs knockdown on mTORC activity, remarkably, it was found that the effect of each HIF knockdown on mTORC activity was opposed: knockdown of HIF-1α (Fig. 4 panel A) resulted in mTORC activity enhancement, visualized as an increase in the phosphorylation of its typical substrate, p70-S6K (P–S6K). Conversely, HIF-2α silencing (Fig. 4 panel B) resulted in mTORC activity inhibition, visualized as a decrease in p70-S6K phosphorylation. Treatment of cells with the combination of CCI-779 and the autophagy flux inhibitor Hydroxychloroquine (HCQ) did not produce significant effects compared with CCI-779 treatment alone, except when cells were depleted of HIF-2α expression. These results agree with those reported by Elorza A et al. [21], which found that activation of the HIF2α pathway in VHL-deficient tumor cells derived from renal cell carcinoma increases mTORC1 activity by upregulating the expression of SLC7A5, an amino acid carrier critical for its activity.

Cancer cells require mTORC1 activity, but mTORC1 hyperactivation would sensitize cells to death by apoptosis [1–3]. Stress granules (SGs) represent important non-membrane cytoplasmic compartments involved in cellular adaptation to stressful conditions such as hypoxia, nutrient deprivation, and oxidative stress allowing cancer cells to escape stress-induced cell death [4]. Notably, Thedieck et al. [7] described Astrin as an essential negative mTORC1 regulator because they showed that Astrin interacts with Raptor recruiting it to SGs to restrict mTORC1 activation upon stress, proposing that mTOR-Raptor dissociation may be a compensatory mechanism to prevent mTORC1 hyperactivation and apoptosis upon stress [7]. However, our results are not consistent with the notion of Astrin as a negative modulator of mTOR activity. Notably, our data indicate that mTORC activity seems to be independent of Astrin expression levels because, as shown in Fig. 3A, HIF-1α expression knockdown greatly increased Astrin expression levels, and despite this,

mTORC activity did not result diminished but on the contrary, displayed high activity when HIF-1α expression was depleted as shown in Fig. 4A. Our data are instead in agreement with recent reports showing that Astrin plays a key role in activating many signaling pathways that are known to be associated with cancer, such as the PI3K/AKT/mTOR signaling pathway [6,22–24].

Altogether our data indicate that it is mTORC that controls both HIFs and Astrin expression connecting them in a survival regulatory mechanism under stress conditions. However, this does not rule out that both Astrin and HIF-1α expression may be part of negative feedback loop regulations to avoid mTORC hyperactivation and apoptosis induction in colon cancer cells. In addition, mTORC also activates a positive loop regulation inducing the expression of HIF-2α, which activates mTORC activity. Thus, there are activating and inhibiting inputs on the mTORC network during different stresses, and the interplay between mTORC, HIFs, and Astrin may adjust the cellular response to them.

Ethics statements

This work does not include any human subjects, animal experiments, or data from social media platforms.

CRediT author statement

Abril Saint-Martin: Conceptualization, Methodology, Data curation, Formal analysis. Marco Antonio Morquecho-León: Methodology, Formal analysis. María Cristina Castañeda-Patlán: data curation, validation, supervision. Martha Robles-Flores: Conceptualization, validation, formal analysis, investigation, Resources, Writing-Review & Editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101336.

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