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Depletion of Amyloid Precursor Protein (APP) causes G0 arrest in non-small cell lung cancer (NSCLC) cells

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Abstract

We recently reported that Amyloid Precursor Protein (APP) regulates global protein synthesis in a variety of human dividing cells, including non-small cell lung cancer (NSCLC) cells. More specifically, APP depletion causes an increase of both cap- and IRES-dependent translation. Since growth and proliferation are tightly coupled processes, here we asked what effects artificial downregulation of APP could have elicited in NSCLC cells proliferation. APP depletion caused a G0/G1 arrest through destabilization of the cyclin-C protein and reduced pRb phosphorylation at residues Ser802/811. siRNA to cyclin-C mirrored the cell cycle distribution observed when silencing APP. Cells arrested in G0/G1 (and with augmented global protein synthesis) increased their size and underwent a necrotic cell death due to cell membrane permeabilization. These phenotypes were reversed by overexpression of the APP C-terminal domain, indicating a novel role for APP in regulating early cell cycle entry decisions. It seems that APP moderates the rate of protein synthesis before the cell clears growth factors- and nutrients-dependent checkpoint in mid G1. Our results raise questions on how such processes interact in the context of (at least) dividing NSCLC cells. The data presented here suggest that APP, although required for G0/G1 transitions, moderates the rate of protein synthesis before the cell fully commits to cell cycle progression following mechanisms, which seem additional to concurrent signals deriving from the PI3-K/Akt/mTORC-1 axis. APP appears to play a central role in regulating cell cycle entry with the rate of protein synthesis; and its loss-of-function causes cell size abnormalities and death.

Keywords

Amyloid Precursor Protein; Cyclin-C; Cell cycle arrest; Non-small cell lung cancer; Cell size

Introduction

G0 exit is regulated by growth factors availability (e.g., serum stimulation; Pardee, 1974). Many mitogens exert their function through stimulation of tyrosine kinase growth factors receptors (RTKs) (Ho et al., 2002). Briefly, RTKs can activate both the Ras/Raf/MAP kinases and the phosphatidylinositol 3-OH kinase (PI3K)/Akt/mTORC-1 signaling pathways (Laplane and Sabatini, 2012). The latter axis profoundly increases anabolic processes,

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including the global rate of protein synthesis. However, it is accepted that the rate of protein accumulation increases linearly during cell cycle progression (Baxter and Stanners, 1978). Hence, growth and proliferation seem to be finely tuned during G1, while the cell clears a growth factor-dependent restriction point (or R) (Zettemberg et al., 1995; Ekholm et al., 2001) and a nutrient-dependent restriction point that some authors have compared to the equivalent of START in yeasts (Fingar and Blenis, 2004). The PI3K/Akt/mTORC-1 axis plays a central role in linking growth and proliferation. Inactivating mutations of several components of this signaling pathway influence both cell size and number, hence organ size (Böhni et al., 1996; Leveers et al., 1996; Goberdan et al., 1999). On the contrary, mutations of genes leading to uncontrolled mTORC-1 activity and failed metabolic checkpoints cause syndromes characterized by multiple tumor-like outgrowths. Some of these syndromes include Cowden syndrome (Liaw et al., 1997) and tuberous sclerosis (Brook-Carter et al., 1994). Finally, knockout of a number of genes responsible for PI3K activation and proper function of its downstream effectors causes organ hypoplasia and reduced body size in mice (Liu et al., 1993; Dummler et al., 2006).

Among the gene knockouts affecting organ and body size, one of the least characterized is Amyloid Precursor Protein, or APP. Mice carrying a homozygous deletion of APP are about 20% smaller than their normal or heterozygous littermates (Zheng et al., 1995). This phenotype has been attributed to a reduced food and liquid intake in APP KO mice.

APP is a single pass transmembrane, highly pleiotropic protein involved in numerous cellular functions, none of which is conclusively considered to be APP's main role (Müller and Zheng, 2012). APP structure and sequential proteolytic cleavage (β - and γ -secretase sequential cleavage) are similar to what is observed in Notch receptors activation, although it is still unclear whether APP is a receptor or a ligand. APP is ubiquitously expressed as multiple splice variants, which undergo extensive post-translational modification. It has two homologues, APLP-1 and APLP-2. APP has been linked to a variety of cellular functions, including iron transport and homeostasis (Duce et al., 2010). Among other functions, the APP C-terminal domain (AICD) seems to affect transcription alongside cofactor Fe65, after its recruitment to membrane-bound APP. Cleavage of APP generates an AICD/Fe65 complex that translocates to the nucleus where it affects transcription alongside factor Tip60 (Cao and Südhof, 2004). A few studies have implicated APP in tumorigenesis; mostly through activation of extracellular signal-regulated protein kinase (Nizzari et al., 2007).

In a previous study we found that APP depletion in human dividing cells of different origin caused a substantial increase in global protein synthesis, both cap- and IRES-dependent under hypoxic conditions (Sobol et al., 2014). Here we wanted to understand what effects could have caused APP depletion in the proliferation of NSCLC cells and normal lung fibroblasts.

Materials and Methods

Unless otherwise specified, all studies are shown 48 hours after experimental manipulation. APP was downregulated using siRNA10 (# SI02780288) from Qiagen (Valencia, CA). Since APP is expressed as ten alternatively spliced variants (whose presence has been

confirmed in all our cells), siRNA10, which targets the very N-terminal portion of the coding sequence, common to all APP splice variants, yields results quantitatively robust. Landmark experiments were confirmed using shRNA to APP cloned in pLKO.1 (#TRCN0000011043 and #TRCN000006707, Sigma-Aldrich, St. Louis, MS). Other siRNAs: ALLStars Negative Control siRNA (Qiagen, # 1027280); Cyclin C (Santa Cruz Biotechnology, # sc-35132).

Reagents

Roscovitine (Cell Signaling, Danvers, MA) was dissolved in DMSO and used at a final concentration of 20 μ M. UO126 (Selleck Chemicals, Huston, TX) was dissolved in DMSO, and used at a final concentration of 10 μ M. MG132 (C2211; Sigma-Aldrich) was dissolved in DMSO and used at a final concentration of 30 μ M. Propidium Iodide (PI; 81845; Sigma-Aldrich) was dissolved at 1 mg/ml in distilled water and used at the concentrations specified below. Acridine Orange (AO; Life Technologies Corp.; Carlsbad, CA) was dissolved in distilled water at a 10 mg/ml concentration and used as specified below. pCDF1-MCS1-EF1-cop GFP expressing the APP C-terminal 59 aa was a gift from Dr. Xiao Z.C. (Institute of Molecular and Cell Biology, Singapore); full length APP 695 in pCAX vector was from Addgene (Cambridge, MA). We used the empty plasmids as transfection controls.

Cell culture and transfection

Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) under 0.5% O₂, 5.0% CO₂, 94.5% N₂ in a Coy CleanSpot glove-box incubator (Coy Laboratory Products, Grass Lake Charter Township, MI). Transfection of siRNA was performed using electroporation as previously described (Chen et al., 2007). Transfection of DNA was performed either using electroporation or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer protocols. Cell lines and cultures were fingerprinted using the GenePrint fluorescent STR system (Promega, Madison, WI). Absence of mycoplasma contamination was monitored using the MycoSensor QPCR assay kit (Stratagene, La Jolla, CA).

Antibodies and protein analysis

We used the following antibodies: rabbit polyclonal anti APP (C-terminal) (Sigma-Aldrich, # A8717); rabbit polyclonal anti Cyclin C (Abcam, Cambridge, MA, # 85927); rabbit polyclonal anti Cyclin E (Santa Cruz Biotechnologies, # 198); mouse monoclonal anti ERK 1/2 (Santa Cruz Biotechnologies, #154); mouse monoclonal anti p-ERK (Cell Signaling, # 05-481); rabbit polyclonal anti Wee1 (Abcam, # 37597); rabbit polyclonal anti p-Wee1 (Ser123) (Abcam, # 60034); mouse monoclonal anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Billerica, MA, # MAB374); goat anti-mouse IgG (H +L), peroxidase-conjugated (Thermo Fisher Scientific Inc., Waltham, MA, # 32430); goat anti-rabbit IgG (H+L), peroxidase conjugated (Thermo Fisher Scientific Inc., # 32460).

Western blot analysis was performed as previously described (Chen et al., 2007). The amount of total cell lysate loaded was experimentally determined to establish a linear range for the assay for each protein assayed (from 5 to 50 μ g total cell lysate). Whenever possible,

nitrocellulose membrane were cut at specific molecular weights and probed simultaneously with different antibodies to reduce artifacts due to multiple stripping of antibodies procedures.

For immunoprecipitation, lysates were incubated with a specific primary antibody at the concentrations recommended by manufacturers overnight at 4 °C, followed by 1 hour incubation with Protein A/G Magnetic Beads (Thermo Fisher Scientific Inc.). Immunoprecipitations were performed according to manufacturer protocols. After elution, proteins were resolved by SDS-PAGE.

Cell Cycle and Cell Cycle Phospho Antibody Microarray were from Full Moon BioSystems (Sunnyvale, CA). 48h after transfection with either control siRNA or siAPP, protein extracts were obtained from cell lines H1299 and A549, and used for the cell cycle-related protein expression profiling. For protein extraction, labeling, conjugation and detection we used the Antibody Array Assay Kit (Full Moon BioSystems) following the manufacturer's instructions. The arrays were scanned and signal quantified by Full Moon BioSystems apparatuses.

Rate of global protein synthesis

The rate of global protein synthesis was measured using the Click-iT AHA (L-Azidohomoalanine) Protein Synthesis kit (Invitrogen) according to the manufacturer's instruction. The incorporation of AHA was analyzed using a BD FACS Canto II instrument (Becton Dickinson, Franklin Lakes, NJ). Flow cytometry data was analyzed by FlowJo software.

For AHA incorporation NSCLC cells were electroporated with siRNA control or siRNA against APP (siAPP), plated in RPMI media supplemented with 10% FBS and allowed to attach overnight. After 36 hours, the medium was changed to medium without methionine, supplemented with 1% FBS. Twelve hours later (48 hours after transfection), cells were handled as per manufacturer instructions. After incubation, cells were detached, washed and analyzed by flow cytometry. Each experiment was performed at least in triplicate.

Cell viability, Cell cycle and proliferation, and acridine orange staining

For cell viability, cells were washed twice in PBS, resuspended in 1 ml of PBS supplemented with 20 µg/ml PI. For cell cycle analysis, cells were washed with 2 ml of 5% FBS, fixed with 70% ethanol and incubated with 250 µl of 10 µg/ml RNase A (Thermo Fisher Scientific Inc.) at 37 °C for 15'. Samples were subsequently incubated at R.T. for 5' and then 250 µl of 100 µg/ml PI was added. For cell proliferation, bromodeoxyuridine (BrdU) incorporation (FITC BrdU Flow kit, BD Pharmingen, San Jose, CA)/7-aminoactinomycin D (7-AAD, Sigma-Aldrich) staining was done according to the manufacturer's instruction. Incorporation of PI or BrdU was acquired and analyzed by flow cytometry.

Differential staining of DNA and RNA was accomplished using Acridine Orange (AO). After transfections, cells were resuspended in RPMI-1640 culture medium supplemented

with 10% FBS. 0.4 ml of permeabilizing solution (0.1% Triton X-100, 80 mM HCl, 150 mM NaCl) was added to 0.2 ml of cell suspension. After 15', 1.2 ml of ice-cold AO staining solution was added (1 mg/ml) and fluorescence was recorded within 2' to 10' using both BD FACS Canto II instrument and Amnis Image Stream X (EMD Millipore Corporation, Billerica, MA).

Gene Expression Analysis

Total RNA was extracted using the RNeasy Mini kit (Qiagen). RNA concentration was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc.) and 1 µg of total RNA was used for cDNA synthesis. cDNA synthesis was performed using iScript Reverse Transcription Supermix RT-qPCR (Bio-Rad, Hercules, CA). Quantitative real-time PCR was done with SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in an ABI 7300 thermal cycler (Applied Biosystems). Non-reverse transcription reactions served as negative controls. All measurements were normalized for the expression of three reference genes mRNAs: human ribosomal protein RPL13A, β -actin, and β -tubulin. We used linearity curves calibrations in all experiments.

Oligonucleotides used in this study are reported in Table 1.

Results

APP depletion causes increased methionine surrogate incorporation

Hypoxic tumor microenvironment is generally considered to be resistant to radio/chemotherapy. Besides being poorly permeable to anticancer drugs, hypoxic tumor, including NSCLC, responds poorly to antiproliferative interventions because it is quiescent compared to well-oxygenated tumor tissue (Kyle et al., 2012). In previous studies, we attempted to specifically target hypoxic NSCLC using γ -secretase inhibitors in order to inhibit Notch-1 signaling both in vitro and in vivo (Chen et al., 2007; Elias et al., 2010; Liang et al., 2012). Reproducing in vitro the conditions found in hypoxic tumor microenvironment is probably impossible, particularly because of its intrinsic heterogeneity, variability and complexity (Palmer et al., 2010). Thus, we performed tissue culture experiments in complete medium, but preserving hypoxia inducible factor 1 α (HIF-1 α) expression in our cells by culturing them under a 0.5% O₂, 5.0% CO₂, 94.5% N₂ atmosphere. In these conditions, cells are not growth or proliferation arrested. At these O₂ concentrations, mitochondrial functions are preserved (Höckel and Vaupel, 2001), therefore 0.5% O₂ is not considered deep hypoxia. Higher oxygen concentrations did not consistently stabilize HIF-1 α (not shown).

During our studies, we serendipitously found that artificial downregulation of the γ -secretase substrate APP caused a considerable increase in the rate of global protein synthesis as measured by the methionine analog AHA incorporation (Fig. 1). This phenomenon has been detailed elsewhere (Sobol et al., 2014). In all cell tested (four NSCLC cell lines, one mesothelioma cell line, in immortalized primary keratinocytes, and in primary lung fibroblasts) we measured an increase in protein synthesis ranging between 27.0% and 164.5% (according to different cell types; Sobol et al., 2014).

APP downregulation causes G0 arrest in NSCLC cells

Regulation of protein synthesis is a crucial biological process linked to proliferation and possibly to the maintenance of cell size homeostasis. Whether the rate of global protein synthesis and cell cycle progression are interlocked processes in mammalian cells is still debated by some. However, it is generally accepted that the blockade of protein synthesis causes cell growth arrest and/or apoptosis (Sluder et al., 1990; Lindqvist et al., 2012), and that excessive protein synthesis can lead to deregulated proliferation (Ruggero and Pandolfi, 2003). Since APP downregulation produced a large increase in the rate of global protein synthesis in a panel of cell lines or cultures, we sought to investigate what effects, if any, this phenomenon may have on the cell cycle. Standard propidium iodide staining of cells at different time-points after siRNA to APP showed a paradoxical increase in the G0/G1 cell population and no appearance of aberrant cell populations (e.g., multiploid or subG0; Fig. 2A and Supplementary Fig. S1). Using BrdU incorporation/FACS analysis, which offers a better representation of cells replicating DNA, we observed a $68.3 \pm 4.4\%$ increase in cells not engaging in DNA replication (H1299 cells; Fig. 2B). Other examples are reported in Supplementary Fig. S2). APP downregulation also caused decreased DNA synthesis in normal lung fibroblasts, albeit to a lower extent compared to the majority of NSCLC cell lines tested (Supplementary Fig. S2). To assess whether cells with depleted APP showed impaired G1 entry, we performed acridine orange (AO) staining/FACS analyses. Using this technique, we assessed that cells with downregulated APP had a 13.8 ± 3.5 fold increase in the G0 fraction compared to controls (Fig. 3A). Albeit AO staining is not the best technique to firmly set a boundary between cells in S and cells in G2/M phase, this approach allowed us to identify a fifth cell population. We named this population “sub-G0” although the cell size (as detected by forward versus side light scattering) was similar to that of cells in G0 or G1. However, this cell population appeared to stain negative for both DNA and RNA. To better understand what this population (virtually absent in control cells) represented, we analyzed these cell populations using an Amnis Image StreamX imaging flow cytometer and determined that these “sub-G0” cells were necrotic, containing no nucleus (Fig. 3B).

These results were entirely unexpected and suggested that APP functions could be necessary to finely modulate cell growth and proliferation early during G0-G1 transition in NSCLC cells under hypoxia.

APP depletion appears to cause G0 arrest through cyclin-C destabilization

Others and we have reported that APP depletion or inhibition interferes with ERKs activation (Russo et al., 2002; Sobol et al., 2014). To explore whether ERK or cyclin dependent kinase (CDK) inhibition was responsible for the cell cycle distribution observed upon APP depletion, we used the MAP-K inhibitor UO126 and Roscovitine, a CDKs inhibitor preferentially targeting CDK-2 and CDK-1 (Shutte et al., 1997). Neither inhibitor recapitulated the effects on the cell cycle shown upon APP depletion: Roscovitine caused a noticeable G2 arrest, while UO126 caused G1 arrest, with no accumulation of cells in G0 (Supplementary Fig. 3).

To identify putative candidates mediating APP effects in our cell systems, we performed antibody arrays that target a large number of proteins and phosphoproteins known to play a

role in the regulation of the cell cycle. Using two cell lines (A549 and H1299), the antibody arrays surprisingly showed only a modest reduction in cyclin-E and a 2.5 fold reduction in cyclin-C upon APP depletion. All other potential players were unchanged after APP downregulation, both in the steady-state expression and phosphorylation levels. We verified the results of the antibody arrays both at the mRNA and protein levels. Q-PCR and Western blot analyses confirmed a 2-fold reduction of both the cyclin-E mRNA and protein 48 hours after APP depletion. A considerable reduction of cyclin-C protein expression levels at all time points after APP downregulation was observed, although the cyclin-C mRNA appeared not to be affected by APP depletion (Fig. 4A and B). Because the pattern of cyclin-C expression (as early as 24 hours after siRNA to APP transfection through 72 hours after transfection) was more consistent with the timing of cell cycle arrest observed upon APP depletion, we mainly focused on this cyclin. Cyclin-C is thought to act in two distinct pools: a fraction of it associates with CDK8 and regulates RNA polymerase II functions (Rickert et al., 1996), while another fraction, extremely labile (Hautbergue and Goguel, 1999) complexes with CDK3 and is necessary for G1 entry (Ren and Rollins 2004). Decreased cyclin-C steady-state expression with unchanged cyclin-C mRNA expression suggested protein destabilization upon APP depletion. We investigated this possibility in immunoprecipitation experiments followed by immunoblotting for cyclin-C and ubiquitin in the absence and in the presence of the proteosomal inhibitor MG132. In the absence of proteosomal inhibitor, we immunoprecipitated about 25% of cyclin-C from lysates obtained from cells transfected with siRNA to APP compared to cells transfected with control siRNA (as determined by densitometry, Fig. 4C). Immunoblot of the same immunoprecipitate using an antibody against ubiquitin showed a more intense smear in immunoprecipitates obtained from siRNA to APP transfected cells compared to cells transfected with control siRNA (Fig. 4D). To confirm that cyclin-C could have been destabilized through the polyubiquitylation/proteosomal degradation system, we treated siRNA transfected cells with MG132 for 24 hours. In these conditions, immunoprecipitation experiments revealed equal amounts of cyclin-C between control and siRNA to APP. The cyclin-C signals were within the margins of variations in electrophoretic mobility due to multiples of ubiquitin molecules (about 8.5 kDa) (Fig. 4E). Although we expected to find cyclin-C at high molecular weights, these results are quite common when long-term proteasomal activity is inhibited and attributed to responses to “ubiquitin depletion” (Kim et al., 2011). Immunoblotting for ubiquitin in these conditions did not allow the identification of defined bands (not shown). Taken together, these results suggest that APP depletion leads to cyclin-C destabilization through the polyubiquitylation/proteasomal degradation pathway.

Cyclin-C/CDK-3 complexes phosphorylate pRB at serines 807/811 to promote G0 exit (Ren and Rollins, 2004). To further confirm that APP depletion leads to G0 arrest through regulation of cyclin-C, we tested pRb phosphorylation at S807/811 upon APP and cyclin-C artificial downregulation (Fig. 4F). Both APP and cyclin-C depletion yielded almost identical results on the status of pRB S807/811 phosphorylation (Fig. 4F). Interestingly, APP depletion caused the specific loss of the cyclin-C isoform that in yeast has been linked to G0 exit (Hautbergue and Goguel, 1999).

Artificial cyclin-C depletion produces a cell cycle distribution qualitatively identical to what is observed when downregulating APP; this phenotype is reversed by APP C-terminal domain forced expression

Artificial downregulation of cyclin-C yielded cell cycle distribution (as measured by AO staining) similar to what was obtained when silencing APP, including the sub-G0 population of necrotic cells (Fig. 5A). It should be noted that siRNA to cyclin-C did not significantly modify the rate of global protein synthesis in all tested cell lines (not shown), hence yielding a sort of uncoupling between the rate of global protein synthesis and cell cycle progression, but not to the same extent of what was obtained when downregulating APP (increased rate of global protein synthesis and cell cycle arrest). This difference may explain the quantitative differences we observed between APP siRNA and cyclin-C siRNA.

Transfection of cells with AICD (59 amino acids long) showed an AO staining pattern qualitatively opposite to APP siRNA (within obvious differences presented by transfecting siRNAs and plasmid DNAs, which appeared to arrest or kill about 25% of transfected cells; Fig. 5B, pCONT). Cells transfected with AICD showed substantially more cells in S and/or G2 compared to cells transfected with control plasmid (Fig. 5B). Also, AICD decreased the fraction of necrotic cells. Hence, the phenotype observed upon APP depletion was mirrored by cyclin-C artificial downregulation and reversed by the forced expression of the APP C-terminal portion.

NSCLC cells with depleted APP increased their size and showed a necrotic phenotype

The data presented above suggested that APP depletion caused a paradoxical dissociation between the rate of protein synthesis and cell cycle progression. Increased synthesis of cellular proteins coupled to cell cycle arrest caused a measurable increase in cell size 72 hours after siRNA transfection (range, 113–130%, Fig. 6A). This phenomenon was paralleled by loss of cell membrane integrity, an event observed by simply exposing cells to propidium iodide without permeabilizing cells, measured by FACS analysis (Fig. 6B). Increased cell death upon APP depletion was also noted in standard forward versus side light scattering during FACS data acquisition (Fig. 6C).

In conclusion, our data show that APP loss-of-function causes complete dissociation between cell growth (which is increased) and cell cycle progression (which is G0 arrested) in NSCLC cells. This dissociation eventually results in cell death.

Discussion

We describe that APP depletion in a variety of NSCLC cells (and, to a lesser extent, in primary lung fibroblasts) causes a cyclin-C dependent cell cycle arrest in G0. This phenotype seems to be reversed by forced expression of the APP C-terminal domain. Paradoxically, cells arrested in G0 display a substantially increased protein synthesis rate (Fig. 1 and Sobol et al., 2014). There is evidence that cell division can be uncoupled with cytoplasmic cell growth and protein synthesis by blocking CDK activity. This occurs either with drugs like staurosporine (Usui et al., 1991) or overexpressing CDK inhibitors like *INK4A* (Ausserlechner et al., 2005). However, these interventions generally lead to large

polyploid cells or G1 arrest with normal protein synthesis rates, respectively. Apoptotic cell death seems to be a common, ultimate outcome when G1 arrest is protracted over several days. Reduced APP expression also seems to interfere with G0/G1 CDK activity through its regulation of cyclin-C (Fig. 4), but this cell cycle arrest is accompanied by a noticeable increase in the rate of global protein synthesis (Fig. 1). This complete uncoupling also leads to cellular abnormalities, such as increased cell volume and cell death. We have observed a necrotic-type cell death, likely due to aberrant cell permeability (Fig. 3 and 6).

We can reconcile the apparent paradoxical results obtained here by proposing that APP, though being necessary for G0/G1 transitions, moderates the rate of protein synthesis before the cell is fully committed to the cell cycle for evident energy preservation purposes (Fig. 7). Alternatively, APP functions could serve as an early modulator of cell size control acting primarily in G0/G1 rather than at the G2/M boundary, as abundantly described elsewhere (Yasutis and Kozminski, 2013). Our data do not address the issue whether a stringent cell size checkpoint in NSCLC cells exists, as previously described in other systems (Conlon et al., 2001; Dolznig et al., 2004). However, they strongly suggest that early mechanisms to coordinate growth and proliferation are in place, and APP seems to play a major role in such process.

Some cells can be grown to different sizes in tissue culture, and since growth and proliferation stimuli largely overlap, a strict mechanism for the establishment of a specific cell size may be unnecessary (Echave et al., 2007). Multiple lines of evidence point to the PI3-K and Myc pathways as crucial nodal points for such a cross-talk. Our data seem to indicate that APP loss-of-function causes increased cell size, but this event appears incompatible with survival, because cell size increase is accompanied by evident compromised cell membrane permeability. This phenomenon can be explained by the observation that increased global protein synthesis upon APP depletion is essentially mTOR-independent (Sobol et al., 2014). Both mTORC-1 and Myc activation stimulate protein synthesis and neolipogenesis (Peterson et al., 2011; Dang, 2011). Although this point needs clarification in future studies, APP may increase protein synthesis without significant neolipogenesis. In this situation, cell membrane homeostasis would be rapidly compromised.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Literature Cited

Ausserlechner MJ, Obexer P, Geley S, Kofler R. G1 arrest by p16INK4A uncouples growth from cell cycle progression in leukemia cells with deregulated cyclin E and c-Myc expression. *Leukemia*. 2005; 19:1051–1057. [PubMed: 15800668]

- Baxter GC, Stanners CP. The effect of protein degradation on cellular growth characteristics. *J Cell Physiol.* 1978; 96:139–145. [PubMed: 670299]
- Böhni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1–4. *Cell.* 1996; 97:865–875. [PubMed: 10399915]
- Brook-Carter PT, Peral B, Ward CJ, Thompson P, Hughes J, Maheshwar MM, Nellist M, Gamble V, Harris PC, Sampson JR. Deletion of the TSC2 and PKD1 genes associated with severe infantile polycystic kidney disease--a contiguous gene syndrome. *Nat Genet.* 1994; 8:328–332. [PubMed: 7894481]
- Cao X, Südhof TC. Dissection of amyloid-beta precursor protein-dependent transcriptional transactivation. *J Biol Chem.* 2004; 279:24601–24611. [PubMed: 15044485]
- Chen Y, De Marco MA, Graziani I, Gazdar AF, Strack PR, Miele L, Bocchetta M. Oxygen concentration determines the biological effects of NOTCH-1 signaling in adenocarcinoma of the lung. *Cancer Res.* 2007; 67:7954–7959. [PubMed: 17804701]
- Conlon IJ, Dunn GA, Mudge AW, Raff MC. Extracellular control of cell size. *Nat Cell Biol.* 2001; 3:918–921. [PubMed: 11584274]
- Dang CV. Therapeutic targeting of Myc-reprogrammed cancer cell metabolism. *Cold Spring Harb Symp Quant Biol.* 2011; 76:369–374. [PubMed: 21960526]
- Dolzign H, Grebien F, Sauer T, Beug H, Müllner EW. Evidence for a size-sensing mechanism in animal cells. *Nat Cell Biol.* 2004; 6:899–905. [PubMed: 15322555]
- Duce JA, Tsatsanis A, Cater MA, James SA, Robb E, Wikke K, Leong SL, Perez K, Johanssen T, Greenough MA, Cho HH, Galatis D, Moir RD, Masters CL, McLean C, Tanzi RE, Cappai R, Barnham KJ, Ciccotosto GD, Rogers JT, Bush AI. Iron-export ferroxidase activity of β -amyloid precursor protein is inhibited by zinc in Alzheimer's disease. *Cell.* 2010; 142:857–867. [PubMed: 20817278]
- Dummler B, Tschopp O, Hynx D, Yang ZZ, Dirnhofer S, Hemmings BA. Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Mol Cell Biol.* 2006; 26:8042–8051. [PubMed: 16923958]
- Echave P, Conlon IJ, Lloyd AC. Cell size regulation in mammalian cells. *Cell Cycle.* 2007; 62:218–224. [PubMed: 17245129]
- Ekholm SV, Zickert P, Reed SI, Zetterberg A. Accumulation of cyclin E is not a prerequisite for passage through the restriction point. *Mol Cell Biol.* 2001; 21:3256–3265. [PubMed: 11287628]
- Elias S, Liang S, Chen Y, De Marco MA, Machek O, Skucha S, Miele L, Bocchetta M. Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene.* 2010; 29:2488–2498. [PubMed: 20154720]
- Fingar DC, Blenis J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene.* 2004; 23:3151–3171. [PubMed: 15094765]
- Goberdhan DC, Paricio N, Goodman EC, Mlodzik M, Wilson C. Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev.* 1999; 13:3244–3258. [PubMed: 10617573]
- Hautbergue G, Goguel V. The yeast C-type cyclin Ctk2p is phosphorylated and rapidly degraded by the ubiquitin-proteasome pathway. *Mol Cell Biol.* 1999; 19:2527–2534. [PubMed: 10082518]
- Ho A, Dowdy SF. Regulation of G91 cell-cycle progression by oncogenes and tumor suppressor genes. *Curr Opin Genet Devel.* 2002; 12:47–52. [PubMed: 11790554]
- Höckel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst.* 2001; 93:266–276. [PubMed: 11181773]
- Kim W1, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, Harper JW, Gygi SP. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell.* 2011; 44:325–340. [PubMed: 21906983]
- Kyle AH, Baker JH, Minchinton AI. Targeting quiescent tumor cells via oxygen and IGF-I supplementation. *Cancer Res.* 2012; 72:801–809. [PubMed: 22158947]
- Laplane M, Sabatini DM. mTOR signaling in growth control and disease. *Cell.* 2012; 149:274–293. [PubMed: 22500797]

- Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD. The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J*. 1996; 15:6584–6594. [PubMed: 8978685]
- Liang S, Galluzzo P, Sobol A, Skucha S, Rambo B, Bocchetta M. Multimodality approaches to treat hypoxic Non-Small Cell Lung Cancer (NSCLC) microenvironment. *Genes Cancer*. 2012; 3:141–151. [PubMed: 23050046]
- Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet*. 1997; 16:64–67. [PubMed: 9140396]
- Lindqvist LM, Vikström I, Chambers JM, McArthur K, Ann Anderson M, Henley KJ, Hoppo L, Cluse L, Johnstone RW, Roberts AW, Kile BT, Croker BA, Burns CJ, Rizzacasa MA, Strasser A, Huang DC. Translation inhibitors induce cell death by multiple mechanisms and Mcl-1 reduction is only a minor contributor. *Cell Death Dis*. 2012; 3:e409. [PubMed: 23059828]
- Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell*. 1993; 75:59–72. [PubMed: 8402901]
- Müller UC, Zheng H. Physiological functions of APP family proteins. *Cold Spring Harb Perspect Med*. 2012; 2:a006288. [PubMed: 22355794]
- Nizzari M, Venezia V, Repetto E, Caorsi V, Magrassi R, Gagliani MC, Carlo P, Florio T, Schettini G, Tacchetti C, Russo T, Diaspro A, Russo C. Amyloid precursor protein and Presenilin1 interact with the adaptor GRB2 and modulate ERK 1,2 signaling. *J Biol Chem*. 2007; 282:13833–13844. [PubMed: 17314098]
- Palmer GM, Fontanella AN, Zhang G, Hanna G, Fraser CL, Dewhirst MW. Optical imaging of tumor hypoxia dynamics. *J Biomed Opt*. 2010; 15:066021. [PubMed: 21198195]
- Pardee AB. A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci U S A*. 1974; 71:1286–1290. [PubMed: 4524638]
- Peterson TR, Sengupta SS, Harris TE, Carmack AE, Kang SA, Balderas E, Guertin DA, Madden KL, Carpenter AE, Finck BN, Sabatini DM. mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell*. 2011; 146:408–420. [PubMed: 21816276]
- Ren S, Rollins BJ. Cyclin C/cdk3 promotes Rb-dependent G0 exit. *Cell*. 2004; 117:239–251. [PubMed: 15084261]
- Rickert P, Seghezzi W, Shanahan F, Cho H, Lees E. Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. *Oncogene*. 1996; 12:2631–2640. [PubMed: 8700522]
- Ruggero D, Pandolfi PP. Does the ribosome translate cancer? *Nat Rev Cancer*. 2003; 3:179–192. [PubMed: 12612653]
- Russo C, Dolcini V, Salis S, Venezia V, Zambrano N, Russo T, Schettini G. Signal transduction through tyrosine-phosphorylated C-terminal fragments of amyloid precursor protein via an enhanced interaction with Shc/Grb2 adaptor proteins in reactive astrocytes of Alzheimer's disease brain. *J Biol Chem*. 2002; 277:35282–35288. [PubMed: 12084708]
- Schutte B, Nieland L, van Engeland M, Henfling ME, Meijer L, Ramaekers FC. The effect of the cyclin-dependent kinase inhibitor olomoucine on cell cycle kinetics. *Exp Cell Res*. 1997; 236:4–15. [PubMed: 9344580]
- Sluder G, Miller FJ, Cole R, Rieder CL. Protein synthesis and the cell cycle: centrosome reproduction in sea urchin eggs is not under translational control. *J Cell Biol*. 1990; 110:2025–2032. [PubMed: 2351692]
- Sobol A, Galluzzo P, Liang S, Rambo B, Skucha S, Weber MJ, Alani S, Bocchetta M. Amyloid Precursor Protein (APP) Affects Global Protein Synthesis in Dividing Human Cells. *J Cell Physiol*. 2014 [Epub ahead of print].
- Usui T, Yoshida M, Abe K, Osada H, Isono K, Beppu T. Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-252a. *J. Cell Biol*. 1991; 115:1275–1282. [PubMed: 1955475]
- Yasutis KM, Kozminski KG. Cell cycle checkpoint regulators reach a zillion. *Cell Cycle*. 2013; 12:1501–1509. [PubMed: 23598718]
- Zetterberg A, Larsson O, Wiman KG. What is the restriction point? *Curr Opin Cell Biol*. 1995; 7:835–842. [PubMed: 8608014]

Zheng H, Jiang M, Trumbauer ME, Sirinathsinghji DJ, Hopkins R, Smith DW, Heavens RP, Dawson GR, Boyce S, Conner MW, Stevens KA, Slunt HH, Sisoda SS, Chen HY, Van der Ploeg LH. beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell*. 1995; 81:525–531. [PubMed: 7758106]

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Fig. 1.

APP depletion causes increased methionine analog AHA incorporation in NSCLC cells; two representative experiments in the indicated cell lines. Dotted lines, cells transfected with control siRNA, solid lines, cells transfected with siRNA to APP. Dashed lines: no AHA negative control (autofluorescence).

Fig. 2.

APP depletion causes cell cycle arrest and decreased DNA synthesis. A: PI staining of A549 cells transfected with a control siRNA (left) and with a siRNA to APP (right). The percentages of cells calculated with the Watson pragmatic model are reported in the figure. Additional examples in H1299 and H1437 cells are reported in supplementary Fig. S1. B: Bromodeoxyuridine (BrdU) incorporation/FACS analysis in H1299 cells transfected with a control siRNA (left) and a siRNA to APP (right). Additional examples in H1437, A549 and in WI38 cells are reported in supplementary Fig. S2.

Fig. 3.

Artificial downregulation of APP causes G0 arrest and necrosis. A: Acridine orange (AO) staining of A549 cells after transfection of the indicated siRNAs. B: Imaging flow cytometer analysis of APP-depleted H1299 cells after siRNA transfection. Left: gating of cell populations; Right: bright field and fluorescence images of representative cells from each cell population. Original magnification 40X. Same results were obtained in A549 and H1650 cells.

Fig. 4.

APP depletion causes cyclin-C destabilization and decreased pRb phosphorylation at S807/811. A: Q-PCR analysis of the indicated mRNAs in A549, H1299 and H1650 transfected with a control siRNA (C) or with a siRNA to APP. Columns represent averages, bars S.D. B: Western blot analysis of the indicated proteins in H1299 cells transfected with control siRNA (C) or siRNA to APP at the indicated time-points. Similar results were obtained in A549, H1650 and H1437 cells. C: Immunoprecipitation of cyclin-C in H1299 transfected with a control siRNA (C) or siRNA to APP. IgG; irrelevant immunoglobulin G.

D: Same as (C), but the membrane was blotted with an antibody against ubiquitin. E: Same as (C), but H1299 cells were treated with the proteasomal inhibitor MG132 for 24 hours prior to immunoprecipitation. F: Western blot analysis of the indicated proteins and phosphoproteins in H1299 cells transfected with a control siRNA (C), with siRNA to APP (siAPP), or with an siRNA against cyclin-C. Similar results were obtained in A549 and H1650 cells.

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Fig. 5.

Artificial downregulation of cyclin-C causes a similar cell cycle distribution compared to APP depletion; these effects are reversed by the APP AICD forced expression. A: Acridine orange (AO) staining of A549 cells after transfection with a control siRNA (C), or with a siRNA to cyclin-C. B: AO staining of A549 cells transfected with a control plasmid (pCont) or with a plasmid expressing AICD. Similar results were obtained in H1299 cells.

Fig. 6.

APP depletion causes increased cell size, cell membrane permeabilization and necrosis. A: Forward light scattering FACS analysis of H1299 cells 72 hours after siRNA transfection. Dotted line, cells transfected with a control siRNA; solid line, cell transfected with siRNA to APP. Similar results were obtained in A549, H1650 and H1437 cells. B: FACS analysis of propidium iodide staining of non-permeabilized A549 cells 72 hours after siRNA transfection. Similar results were obtained in H1299 and H1650 cells. C: Gating strategy of cells shown in (A). Please note a distinct population, which has lost forward light scattering, hence cellular content.

Fig. 7.

Brief schematic of APP functions during G₀/G₁ transitions. The triggering event is universally recognized to be growth factor stimulation. APP participates to G₁ entry by preserving adequate amounts of cyclin-C. Growth factor stimulation also causes over-activation of mTORC-1. This could lead to exacerbated global protein synthesis in stages where the cell has not yet committed to cell division. APP seems to moderate protein synthesis during G₁ entry via an mTOR-independent mechanism (Sobol et al., 2014).

Table 1

List of oligonucleotides used in this study.

Gene	oligonucleotide sequence
Cyclin E	Fw 5'-CAGATTGCAGAGCTGTTGGA-3' Rev 5'-TCCCCGTCTCCCTTATAACC-3'
Cyclin C	Fw 5'-CCAGTATGTGCAGGACATGG-3' Rev 5'-TCCACAGAAAGCTCAGCAAA-3'
RPL13	Fw 5'-CATAGGAAGCTGGGAGCAAG-3' Rev 5'-ACAAGATAGGGCCCTCCAAT-3'
β -tubulin	Fw 5'-ACCTTCAGTGTGGTGCCTTC-3' Rev 5'-GTGGCTGAGACAAGGTGGTT-3'
β -actin	Fw 5'-TCCCTGGAGAAGAGCTACGA-3' Rev 5'-AGCACTGTGTGGCGTACAG-3'
APP splice variants	5'-GCCAAAGAGACATGCAGTGA-3' 5'-CCAGACATCCGAGTCATCCT-3' 5'-CACAGAGAGAACCACCAGCA-3' 5'-CTTGACGTTCTGCCTCTTCC-3' 5'-TTCCCGTGAATGGAGAGTTC-3' 5'-CTCCACCACACCATGATGAA-3' 5'-CCGCTGGTACTTTGATGTGA-3' 5'-GGCAAGAGGTTCTGGGTAG-3' 5'-CACAGAGAGAACCACCAGCA-3' 5'-ACATCCGCCGTAAGAAGATG-3' 5'-CTTGCTGTGCGTGGTAGAAG-3' 5'-TCCATTGAATCTGGGGAAG-3' 5'-GCTGGAGGTCTACCCTGAACT-3' 5'-GCTGCTGTTGTAGGAACTCG-3' 5'-CTCTCCCTCCCCTGTTACG-3' 5'-CTGGTTGGTTGGCTTCTACC-3'