

# The AMPK stress response pathway mediates anoikis resistance through inhibition of mTOR and suppression of protein synthesis

TL Ng<sup>1,2</sup>, G Leprivier<sup>1</sup>, MD Robertson<sup>1</sup>, C Chow<sup>1</sup>, MJ Martin<sup>3</sup>, KR Laderoute<sup>4</sup>, E Davicioni<sup>5</sup>, TJ Triche<sup>6</sup> and PHB Sorensen<sup>\*,1,2</sup>

Suppression of *anoikis* after detachment of cancer cells from the extracellular matrix is a key step during metastasis. Here we show that, after detachment, mouse embryonic fibroblasts (MEFs) transformed by K-Ras(V12) or ETV6-NTRK3 (EN) activate a transcriptional response overrepresented by genes related to bioenergetic stress and the AMP-activated protein kinase (AMPK) energy-sensing pathway. Accordingly, AMPK is activated in both transformed and non-transformed cells after detachment, and AMPK deficiency restores *anoikis* to transformed MEFs. However, AMPK activation represses the mTOR complex-1 (mTORC1) pathway only in transformed cells, suggesting a key role for AMPK-mediated mTORC1 inhibition in the suppression of *anoikis*. Consistent with this, *AMPK*<sup>-/-</sup> MEFs transformed by EN or K-Ras show sustained mTORC1 activation after detachment and fail to suppress *anoikis*. Transformed *TSC1*<sup>-/-</sup> MEFs, which are incapable of suppressing mTORC1, also undergo *anoikis* after detachment, which is reversed by mTORC1 inhibitors. Furthermore, transformed *AMPK*<sup>-/-</sup> and *TSC1*<sup>-/-</sup> MEFs both have higher total protein synthesis rates than wild-type controls, and translation inhibition using cycloheximide partially restores their *anoikis* resistance, indicating a mechanism whereby mTORC1 inhibition suppresses *anoikis*. Finally, breast carcinoma cell lines show similar detachment-induced AMPK/mTORC1 activation and restoration of *anoikis* by AMPK inhibition. Our data implicate AMPK-mediated mTORC1 inhibition and suppression of protein synthesis as a means for bioenergetic conservation during detachment, thus promoting *anoikis* resistance.

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*Anoikis* refers to the cell death that normal non-hematopoietic cells undergo when they become detached from their native extracellular matrix.<sup>1</sup> Cancer cells, by contrast, are able to suppress *anoikis*, allowing them to survive under anchorage-independent conditions such as in the circulation, lymphatics, or bone marrow. This is thought to be a critical step prior to the development of overt metastases.<sup>2</sup> To model *anoikis* resistance *in vitro*, cell lines can be cultured under suspension conditions by preventing attachment to the cell culture substratum. Under such conditions, non-transformed cells undergo rapid apoptosis whereas transformed cells survive indefinitely after detachment.<sup>3</sup> *Anoikis* resistance *in vitro* correlates robustly with *in vivo* metastasis after intravascular injection into the tail veins of immunodeficient mice.<sup>4</sup> Therefore, *anoikis* resistance represents a unique metastasis-promoting mechanism, and a novel anti-metastasis therapeutic target.

Most studies on *anoikis* resistance have focused on kinases directly modulating the apoptosis machinery, such as FAK, TrkB, and EGFR, after detachment.<sup>4–7</sup> Moreover, oncogenic kinases such as the ETV6-NTRK3 (EN) chimeric tyrosine

kinase confer *anoikis* resistance.<sup>8</sup> EN fails to transform mouse embryonic fibroblasts (MEFs) lacking IGF1R (R<sup>-</sup> cells), which correlates with an inability of EN to suppress *anoikis* or activate the PI3K–Akt pathway after detachment unless IGF1R is re-expressed (R<sup>+</sup> cells).<sup>8</sup> Interestingly, a myristoylated, constitutively active form of EN (ENmyr) transforms and suppresses *anoikis* in R<sup>-</sup> cells.<sup>8</sup> These and other data point to a role for IGF1R and PI3K–Akt in *anoikis* resistance.<sup>6</sup> Kinase activation also induces pro-survival pathways, including Ras-ERK,<sup>9</sup> to downregulate pro-apoptotic Bim<sup>5</sup> and upregulate anti-apoptotic Bcl-2.<sup>3</sup> However, mechanisms that promote *anoikis* resistance other than by directly suppressing apoptosis are unclear.

Recently, mechanisms affecting cellular bioenergetic status have been implicated in *anoikis* suppression. Mammary epithelial cells activate macroautophagy in response to detachment to suppress *anoikis*.<sup>10</sup> Detached non-transformed cells have reduced ATP levels, and oncogenic transformation reduces energy stress through modulation of glucose uptake and reactive oxygen species.<sup>11</sup> Detached cells also demonstrate increased endoplasmic reticulum stress owing to

<sup>1</sup>Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, BC, Canada V5Z 1L3; <sup>2</sup>Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada V6T 1Z4; <sup>3</sup>Cancer Research UK Tumour Cell Signalling Unit, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK; <sup>4</sup>Biosciences Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA; <sup>5</sup>GenomeDx Biosciences Inc., 1595 W. 3rd Avenue, Vancouver, BC, Canada V6J 1J8 and <sup>6</sup>Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, CA 90027, USA \*Corresponding author: PHB Sorensen, Department of Molecular Oncology, British Columbia Cancer Research Centre, Room 3.302, 675 W. 10th Avenue, Vancouver, BC, Canada V5Z 1L3. Tel: +1 604 675 8202; Fax: +1 604 675 8218; E-mail: psor@interchange.ubc.ca

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**Abbreviations:** AMPK, AMP-activated protein kinase; CHX, cycloheximide; DN, dominant negative; EN, ETV6-NTRK3; FACS, fluorescence-activated cell sorting; GEP, gene expression profile; GO, gene ontology; GSEA, Gene Set Enrichment Analysis; LDH, lactate dehydrogenase; MEF, mouse embryonic fibroblast; mTORC1, mTOR complex-1; TSC, tuberous sclerosis complex; *wt*, wild type.

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activation of PERK and induction of HIF1 $\alpha$  to promote *anoikis* resistance.<sup>12,13</sup> These studies suggest that detached cells are bioenergetically compromised and activate stress response pathways as a compensatory mechanism.

Here we investigated *anoikis* resistance in transformed cells driven by oncoproteins known to suppress *anoikis*, namely EN and oncogenic K-Ras. We show that detachment results in a broad transcriptional response and modulation of prototypical cellular stress pathways. One such cascade identified is the AMP-activated protein kinase (AMPK) pathway, which is activated under multiple stress conditions.<sup>14</sup> We show that *anoikis* resistance in transformed cells strongly correlates with and is dependent on AMPK activation. Moreover, AMPK-dependent mTOR complex-1 (mTORC1) blockade and inhibition of energy-demanding protein synthesis are critical for *anoikis* suppression, through mitigation of the metabolic defects induced by detachment. Overall, we show that detachment is a bona fide form of cellular stress, and that subsequent survival is dependent on stress response processes typically considered tumor-suppressive, namely AMPK activation and mTOR inhibition. We propose that this represents a further example of 'non-oncogene addiction', whereby cancer cells require a robust stress response to survive transient stresses such as cellular detachment.<sup>15</sup>

## Results

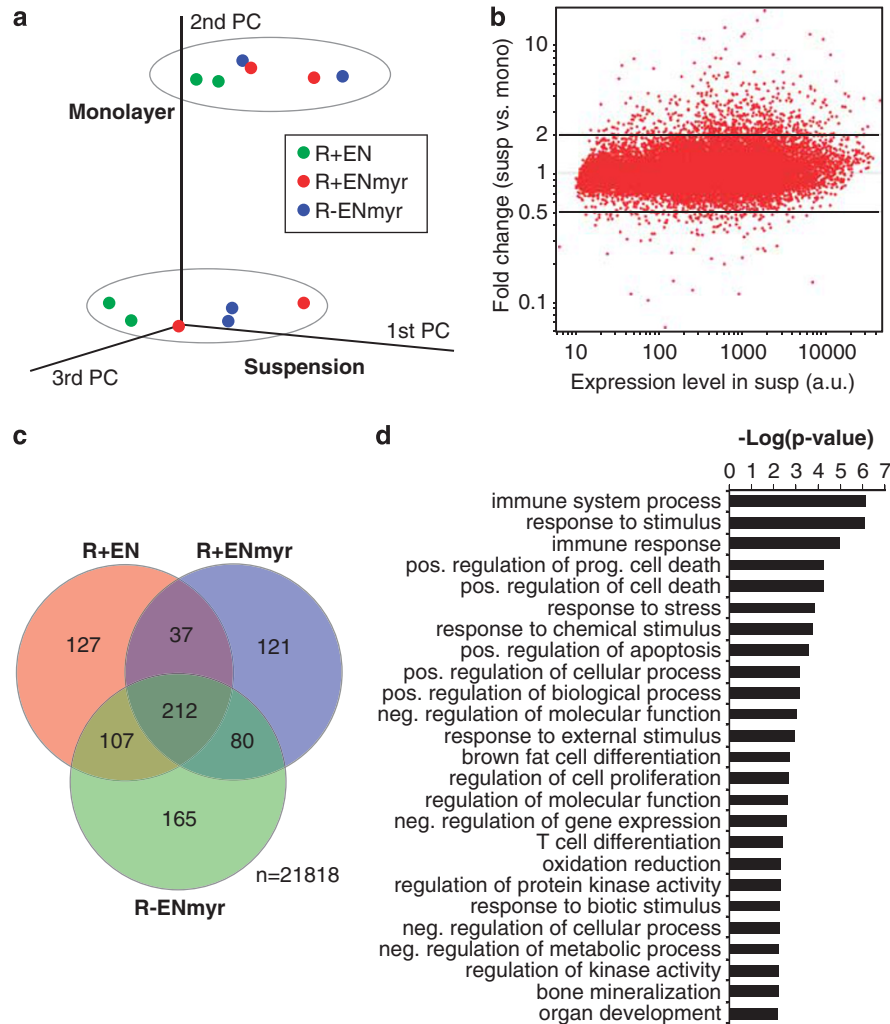
**Transformed fibroblasts activate multiple stress responses during detachment-induced stress.** To model *anoikis* suppression we used NIH3T3 and MEF cell lines stably expressing the oncogenes EN and K-Ras(V12), each previously shown to suppress *anoikis*.<sup>3,8</sup> We first performed gene expression profiling to compare detached (suspension) cultures of EN-transformed MEFs *versus* corresponding monolayer cultures. Three cell line models were used to avoid cell line-specific effects, including R<sup>-</sup> cells expressing ENmyr and R<sup>+</sup> cells expressing either EN or ENmyr. As mentioned, EN cannot transform R<sup>-</sup> cells unless IGF1R is re-expressed, whereas ENmyr transforms R<sup>-</sup> or R<sup>+</sup> cells, and suppresses *anoikis* in both.<sup>8</sup> Principal-component analysis of the resulting gene expression profiles (GEPs) demonstrated detachment as a major source of variation in gene expression (Figure 1a and Supplementary Figure S1c). Contribution of either cell line type or specific EN construct did not feature prominently in any of the first three principal components (Supplementary Figures S1a and b). A large number of genes were differentially expressed in suspension *versus* monolayer cultures (Figure 1b). Overall, we detected more differentially expressed genes with higher fold change in suspension ('upregulated') than in monolayer cultures ('downregulated'). When the three different cell lines were analyzed individually, there was considerable overlap in upregulated genes for each cell line in suspension, with a core of 212 probesets (corresponding to 170 genes) that were significantly upregulated >2-fold in all three cell lines (Figure 1c and Supplementary Table S1a; equivalently >2-fold downregulated genes in Supplementary Table S1b).

Gene ontology (GO) overrepresentation analysis of these 170 genes showed significant enrichment for functional

categories relating to cell death (Figure 1d). In addition, there was enrichment for diverse categories relating to cellular stress; for example, under the GO category of 'response to stress', many upregulated genes were prototypical stress response genes such as *Gadd45a*, *Bnip3*, *Cdkn1*, and *Ddit3*. Importantly, several enriched categories were specifically related to negative regulation of cellular processes such as 'gene expression' and 'metabolic process'. We therefore hypothesized that detachment of transformed cells triggers a significant stress response associated with the attenuation of bioenergetically rich processes. To better characterize the observed stress responses, we used a publicly available data set of annotated microarray experiments ('Chemical and Genetic Perturbations' data set; Broad Institute) to analyze the detachment-induced GEPs of EN-transformed cells by Gene Set Enrichment Analysis (GSEA). Among the most significantly enriched gene sets in detached cells were signatures previously linked to stress conditions such as hypoxia, nutrient and amino-acid deprivation, and epithelial-to-mesenchymal transition (Supplementary Tables S2a and b). Similar specific stress categories were observed among downregulated genes (e.g., see 'peng\_leucine\_up' *versus* 'peng\_leucine\_dn', and 'manalo\_hypoxia\_up' *versus* 'manalo\_hypoxia\_dn'). Overall, the transcriptional profiles observed after detachment mimic diverse forms of cellular stress, suggesting that detachment induces multiple stress responses.

Given the above GEP results, we wondered whether a specific signaling pathway might underlie this broad stress response. One pathway known to negatively modulate diverse cellular functions under stress conditions is the AMP-activated protein kinase (AMPK) cascade, which suppresses multiple energy-demanding functions such as proliferation and protein translation in response to reduced ATP/AMP ratios.<sup>16</sup> To explore this possibility, we cross-compared detachment-induced GEPs with published transcriptional responses observed after modulation of the AMPK pathway (Supplementary Tables S3a–c).<sup>17–19</sup> By GSEA, detachment-induced GEPs showed marked enrichment for genes known to be downregulated after AMPK inhibition and upregulated after AMPK activation<sup>17–19</sup> (Figure 2a). Prominent were genes previously ascribed to AMPK activation, including downregulation of *FASN* and *G6Pase* and upregulation of *p21/Cip1* and *cyclin-G* (Supplementary Tables S1a and b).<sup>14,16</sup> These data support the notion that the transcriptional response to detachment mirrors that of AMPK activation.

**AMPK is activated during detachment-induced stress and promotes resistance to *anoikis*.** We next investigated whether the AMPK pathway is biochemically activated in response to detachment. Using NIH3T3 fibroblasts transformed with EN or K-Ras *versus* MSCV vector alone, the known AMPK targets ACC1 and Raptor, as well as AMPK itself, showed rapid and sustained phosphorylation after detachment (Figure 2b). As this was observed in both transformed and non-transformed cells, AMPK activation alone is likely insufficient for the survival of detached cells. To specifically test whether AMPK activation is critical for *anoikis* resistance in transformed cells, we expressed EN, K-Ras, or the vector alone in wild-type (*wt*) (*AMPK*<sup>+/+</sup>) *versus*

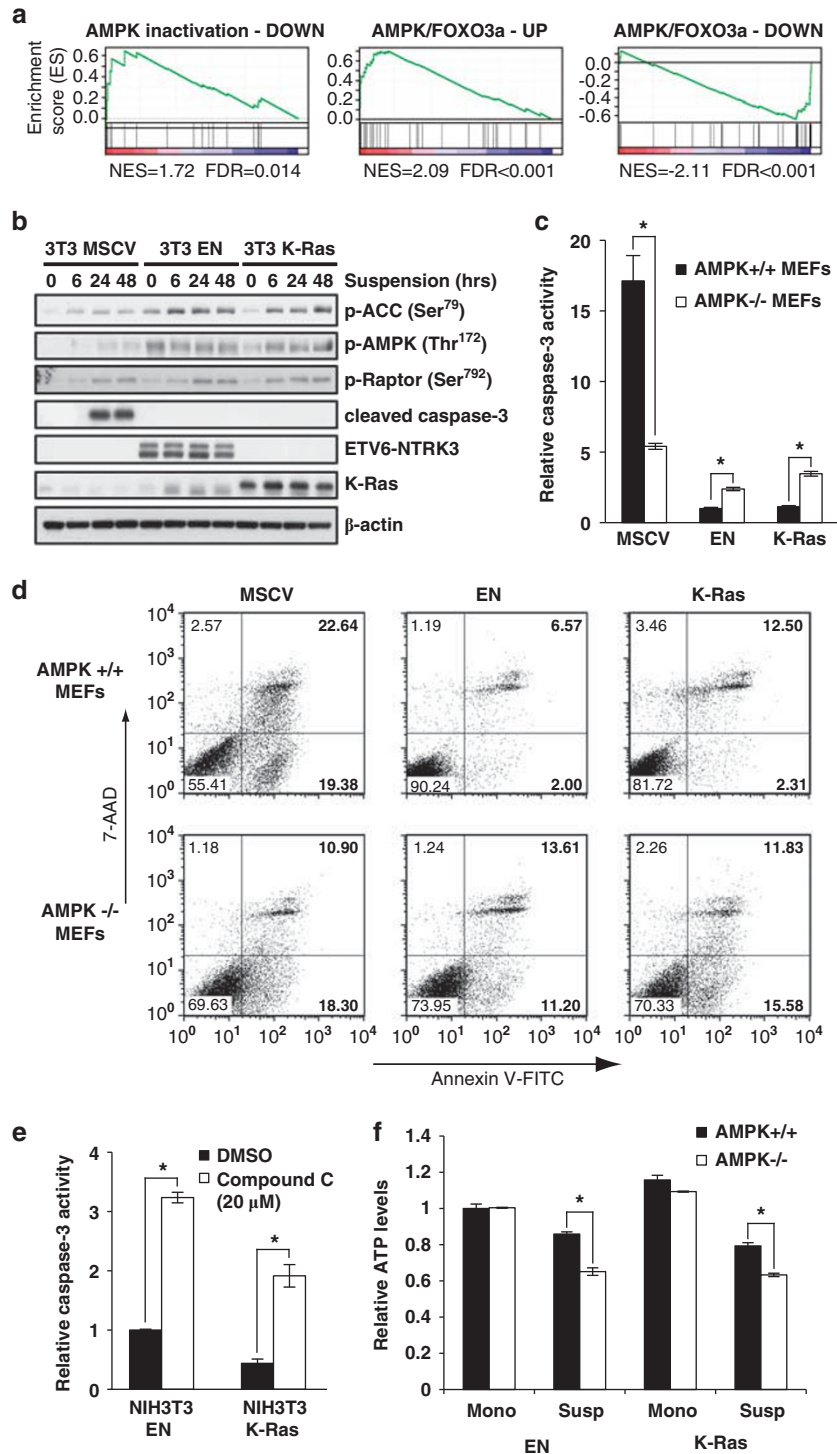


**Figure 1** Cellular detachment induces a multi-faceted transcriptional pattern resembling diverse bioenergetic stress responses. (a) Principal-component analysis illustrating the three largest sources of variation in GEPs, showing clustering of samples based primarily on culture conditions (suspension versus monolayer). (b) Scatter-plot summarizing the mean fold changes of individual genes in suspension as compared with monolayer culture (y-axis) as a function of the mean expression of each gene in all suspension culture samples (x-axis). (c) Venn diagram depicting the overlap of upregulated gene probesets in suspension for the indicated EN-transformed cell lines used in the microarray analysis. (d) GO biological process categories overrepresented in the 170 genes upregulated after detachment in all three EN-transformed cell lines (Supplementary Table S1), for all categories with FDR < 0.10. The x-axis represents the negative log of the DAVID significance score *P*-values

*AMPK $\alpha$ 1* and *AMPK $\alpha$ 2* double-knockout (DKO) (*AMPK*<sup>-/-</sup>) MEFs, and subjected the cells to detachment. Cell death was significantly increased in all *AMPK*<sup>-/-</sup> as compared with *AMPK*<sup>+/+</sup> cells despite EN or K-Ras transformation, as measured by caspase-3 cleavage (Figure 2c). This corresponded closely to apoptosis as measured by annexin-V/7-aminoactinomycin-D (7-AAD) fluorescence assisted cell-sorting (FACS), with increased annexin-V positivity in *AMPK*<sup>-/-</sup> versus *AMPK*<sup>+/+</sup> cells, in both EN-transformed (24.81 versus 8.57%) and K-Ras-transformed (27.41 versus 14.81%) MEFs (Figure 2d). Similar findings were obtained by using the AlamarBlue cell survival assay (Supplementary Figure S2a) and in two different cytotoxicity assays, namely lactate dehydrogenase (LDH) release (Supplementary Figure S2b) and Cytotox-Glo dead cell protease release (Supplementary Figure S2c). The results were validated in EN- or K-Ras-transformed NIH3T3 cells by

chemical inhibition of AMPK using compound-C (Figure 2e), or by molecular inhibition using dominant-negative (DN) AMPK (Supplementary Figure S2d), each of which partially restored *anoikis*.

As AMPK is activated by reduced ATP/AMP ratios,<sup>16</sup> we next compared the ATP levels of the transformed cell lines after detachment. EN- or K-Ras-transformed *AMPK*<sup>+/+</sup> and *AMPK*<sup>-/-</sup> MEFs both showed reduced ATP levels after detachment (Figure 2f). Therefore the decrease in ATP appears proximal to AMPK activation in transformed cells subjected to detachment.<sup>11</sup> However, the ATP decrease was significantly more pronounced in *AMPK*-deficient cells as compared with corresponding *wt* cells, suggesting that AMPK activation functions to maintain ATP levels after detachment. Together, these data demonstrate that AMPK activation promotes survival during detachment-induced stress, possibly by mitigating ATP reduction after detachment.



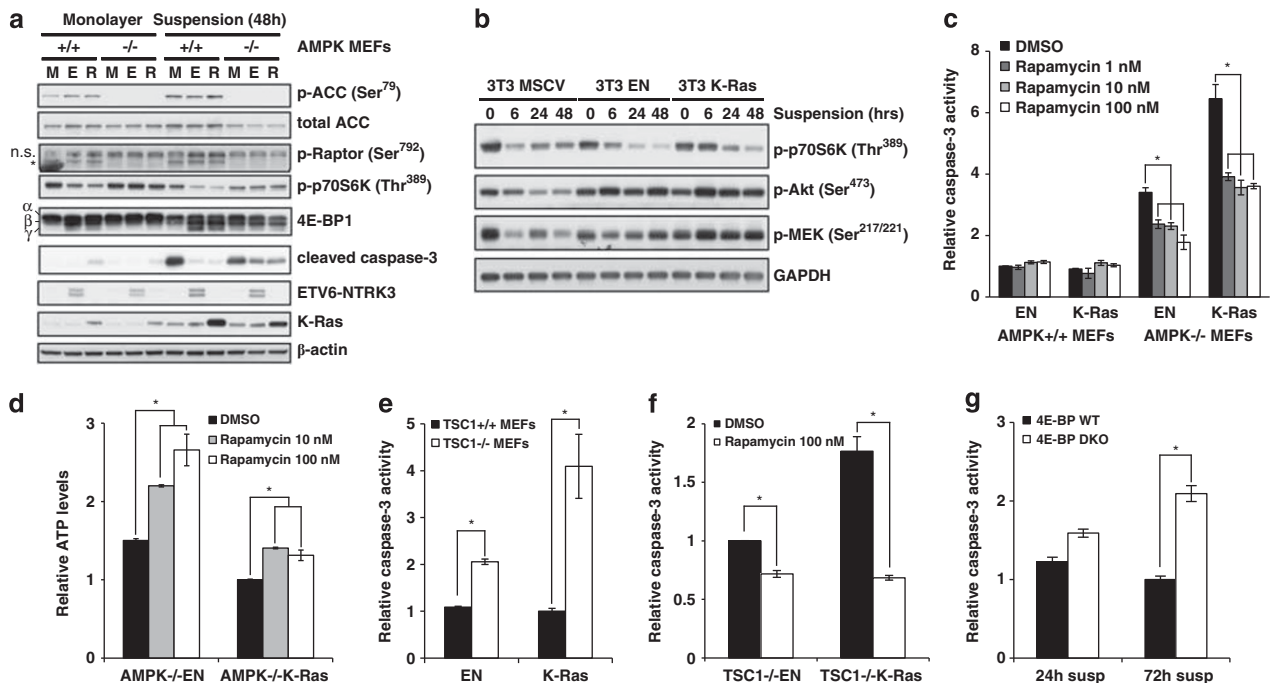
**Figure 2** The AMPK pathway is activated after cellular detachment and promotes *anoikis* resistance. (a) GSEA analysis of detachment-induced GEPs using annotated data sets from the literature of genes known to be altered after AMPK pathway modulation. (b) Western blot demonstrating levels of p-ACC, p-Raptor, and p-AMPK as readouts of AMPK pathway activation at various time points after detachment of transformed and non-transformed NIH3T3 cells. EN and K-Ras(V12) levels are shown by NTRK3 and K-Ras immunoblotting, respectively. Cleaved caspase-3 immunoblotting demonstrates levels of apoptosis in transformed *versus* non-transformed cells.  $\beta$ -Actin is used as a loading control. (c and d) Caspase-3 activity assay and annexin-V/7-AAD FACS analysis of MSCV vector-alone-, EN-, or K-Ras-transformed AMPK<sup>+/+</sup> *versus* AMPK<sup>-/-</sup> MEFs after 48 h in suspension. (e) Caspase-3 activity assay of EN- or K-Ras-transformed NIH3T3 cells treated with compound-C immediately after detachment and cultured in suspension for 48 h. (f) ATP assay of EN- or K-Ras-transformed AMPK<sup>+/+</sup> *versus* AMPK<sup>-/-</sup> MEFs following 24 h in suspension. All data are shown as mean  $\pm$  S.E.M. ( $n=3$ ). The asterisk indicates statistical significance as determined by Student's *t*-test ( $P<0.05$ )



**AMPK activation after detachment inhibits mTORC1 to promote *anoikis* resistance in transformed cells.** AMPK modulates numerous downstream targets to maintain survival during energy stress, including ACC1, eEF2K, mTORC1, and p53.<sup>16</sup> We next wished to determine which pathways downstream from AMPK activation act to suppress *anoikis*. Consistent with NIH3T3 cells, ACC1 and Raptor show increased phosphorylation in suspension *versus* monolayer cultures of *AMPK*<sup>+/+</sup> MEFs, whereas, as expected, no phosphorylation was seen in *AMPK*<sup>-/-</sup> MEFs (Figure 3a). Again, no significant difference was seen between transformed and non-transformed cells. However, we found that survival after detachment in transformed *AMPK*<sup>+/+</sup>/EN or *AMPK*<sup>+/+</sup>/K-Ras MEFs strongly correlated with mTORC1 inhibition, as demonstrated by hypo-phosphorylation of p70S6K and 4E-BP1 (Figure 3a). By contrast, all *AMPK*<sup>-/-</sup> MEF lines showed sustained mTORC1 activity in suspension. A causative role for AMPK activation was corroborated by using either DN AMPK expression (Supplementary Figure S3a) or compound-C treatment of NIH3T3 cells (Supplementary Figure S3b), each of which prevented mTORC1 suppression after detachment (Supplementary Figure S3b). In addition, *anoikis*-sensitive *AMPK*<sup>+/+</sup>/MSCV MEFs also maintained mTORC1 activation, suggesting that,

paradoxically, AMPK-mediated mTORC1 suppression is dependent on oncogenic transformation by EN or K-Ras. Hence, under detachment-induced stress, mTORC1 activity is uncoupled from the hyper-activation of PI3K–Akt and Ras–ERK normally driven by EN or K-Ras. Transformed NIH3T3 cells similarly suppressed mTORC1 activity, in contrast to NIH3T3-MSCV cells, which maintained mTORC1 activity after detachment, again demonstrating that *anoikis* resistance correlates with mTORC1 suppression (Figure 3b). The latter was again uncoupled from PI3K–Akt and Ras–ERK hyper-activation in transformed cells, as high p-Akt and p-MEK levels were sustained even after 48 h in suspension. This further supports the notion that AMPK-mediated mTORC1 inhibition is somehow dependent on oncogenic transformation.

We then wished to establish a direct role for mTORC1 inhibition in *anoikis* resistance. First, *AMPK*<sup>-/-</sup>/EN and *AMPK*<sup>-/-</sup>/K-Ras MEFs, neither of which suppress mTORC1 activity under detachment, were treated with rapamycin immediately after detachment. Rapamycin reduced cell death in transformed *AMPK*<sup>-/-</sup> cells in a dose-dependent manner (Figure 3c), validated in Cytotox-Glo cytotoxicity assays (Supplementary Figure S3d). Rapamycin also restored ATP levels (Figure 3d), which were otherwise significantly reduced after detachment (Figure 2f). To support these findings, we

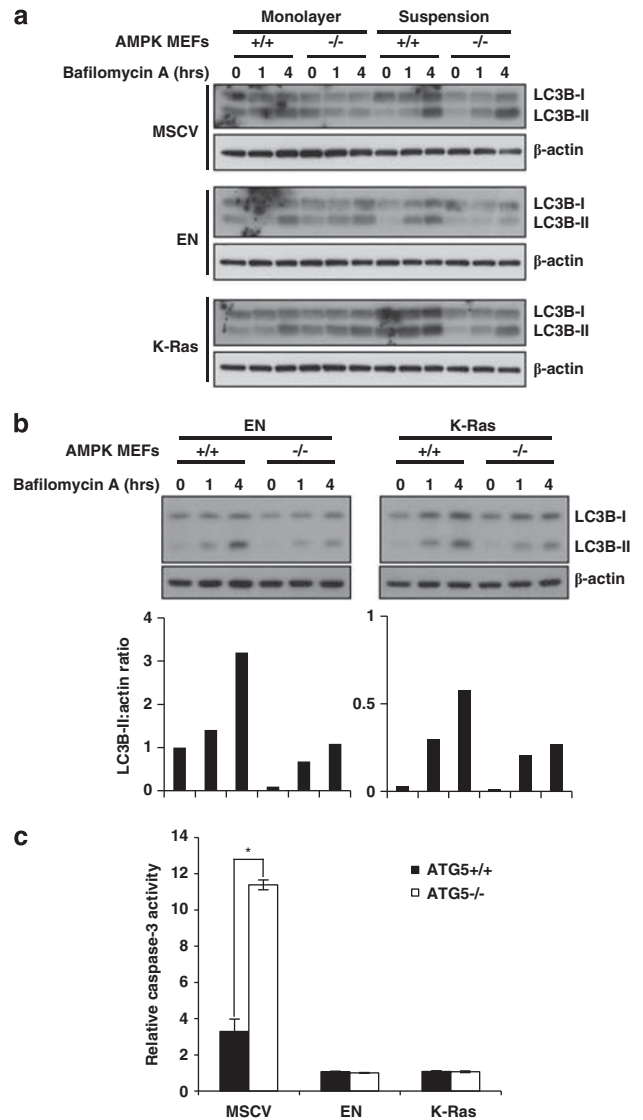


**Figure 3** mTORC1 pathway inhibition is dependent on both AMPK activation and oncogenic transformation, and promotes *anoikis* resistance. (a) Western blot of transformed and non-transformed *AMPK*<sup>+/+</sup> *versus* *AMPK*<sup>-/-</sup> MEFs 48 h after plating as monolayer or suspension cultures, using antibodies against the indicated proteins as pathway readouts for AMPK (p-ACC and p-Raptor) and mTORC1 (p-p70S6K and 4E-BP1). EN and K-Ras(V12) levels are demonstrated as in Figure 2b; cleaved caspase-3 levels are shown to reflect levels of apoptosis; and β-actin is used as a loading control. (b) Western blot of NIH3T3 cells expressing MSCV vector alone *versus* EN or K-Ras(V12) demonstrating the indicated readouts of mTORC1 (p-p70S6K), PI3K-Akt (p-Akt), and Ras-ERK (p-MEK) pathway activities at various time points after detachment. GAPDH is used as a loading control. (c) Caspase-3 activity assay of EN- or K-Ras-transformed *AMPK*<sup>+/+</sup> *versus* *AMPK*<sup>-/-</sup> MEFs treated with a dose-response curve of rapamycin immediately after detachment, and cultured in suspension for 48 h. (d) ATP assay of rapamycin-treated, EN- or K-Ras-expressing *AMPK*<sup>-/-</sup> MEFs after 24 h in suspension. (e) Caspase-3 activity assay of EN- or K-Ras-transformed *TSC1*<sup>+/+</sup> *versus* *TSC1*<sup>-/-</sup> MEFs subjected to 48 h in suspension. (f) Caspase-3 activity assay of EN- or K-Ras-expressing *TSC1*<sup>-/-</sup> MEFs treated with rapamycin and subjected to 48 h in suspension. (g) Caspase-3 activity assay of *4E-BP1* wt *versus* DKO MEFs expressing E1A/Ras at 24 and 72 h after detachment. All data are shown as mean ± S.E.M. (*n* = 3). The asterisk indicates statistical significance as determined by Student's *t*-test (*P* < 0.05).

used *TSC1*<sup>-/-</sup> MEFs, which show constitutive mTORC1 activation.<sup>20</sup> Detached EN and K-Ras transformed *TSC1*<sup>-/-</sup> MEFs indeed showed increased mTORC1 activity after detachment compared with corresponding *TSC1*<sup>+/+</sup> MEFs (Supplementary Figure S3c). Like *AMPK*<sup>-/-</sup> MEFs, *TSC1*<sup>-/-</sup> MEFs showed dramatically increased cell death relative to corresponding *wt* MEFs (Figure 3e), as well as reduced cell survival (Supplementary Figure S3e) and increased cytotoxicity (Supplementary Figures S3f and g). Furthermore, rapamycin again partially suppressed *anoikis* in *TSC1*<sup>-/-</sup>/EN and *TSC1*<sup>-/-</sup>/K-Ras MEFs (Figure 3f and Supplementary Figure S3h). Similar experiments were then performed using MEFs deficient in both 4E-BP1 and 4E-BP2 (downstream from mTORC1/p70S6K) transformed with E1A and H-Ras (*4E-BP* DKO E1A/Ras MEFs), which lack the inhibitory control of protein translation.<sup>21</sup> Indeed, by 72 h in suspension, *4E-BP* DKO E1A/Ras MEFs were markedly more susceptible to *anoikis* as compared with corresponding *wt* MEFs (Figure 3g). Altogether, these data establish a critical role for AMPK-mediated mTORC1 inhibition in the *anoikis* resistance of transformed cells.

#### Loss of ATG5-dependent autophagy is insufficient to restore *anoikis* susceptibility to transformed cells.

Inhibition of mTORC1 induces multiple other processes that could contribute to *anoikis* resistance, including induction of macroautophagy. It was reported previously that macroautophagy is induced after detachment and has a pro-survival role in detached, non-transformed or Bcl-2-transformed breast cells.<sup>10</sup> Consistent with this, both transformed and non-transformed *AMPK*<sup>+/+</sup> MEFs showed increased macroautophagy in suspension cultures, with increased LC3B-II levels after bafilomycin-A-mediated lysosomal inhibition to monitor autophagic flux (Figure 4a). Interestingly, whereas bafilomycin-A-treated *AMPK*<sup>-/-</sup> MEFs also accumulate LC3B-II after detachment, transformed *AMPK*<sup>-/-</sup> MEFs accumulated less LC3B-II compared with the corresponding transformed *AMPK*<sup>+/+</sup> MEFs (Figure 4b). Although mTORC1 is known to block the proximal steps of macroautophagy,<sup>22</sup> the inability of transformed *AMPK*<sup>-/-</sup> MEFs to induce autophagic flux as compared with non-transformed *AMPK*<sup>-/-</sup> control cells is unlikely due to differences in mTORC1 activity, as mTORC1 is re-activated in both transformed and non-transformed *AMPK*<sup>-/-</sup> MEFs in suspension (Figure 3a). Therefore our results are inconsistent with macroautophagy alone as the basis of *anoikis* resistance, as both transformed and non-transformed *AMPK*<sup>-/-</sup> MEFs undergo *anoikis* in suspension (Figure 4a). To more directly demonstrate this, we tested whether defective autophagy restores *anoikis* to transformed cells. Under detachment, we observed a significant increase in cell death of autophagy-deficient, non-transformed *ATG5*<sup>-/-</sup> versus *ATG5*<sup>+/+</sup> MEFs (Figure 4c), consistent with previous reports.<sup>10</sup> By contrast, EN or K-Ras expression almost completely abrogated *anoikis* in both *ATG5*<sup>+/+</sup> and *ATG5*<sup>-/-</sup> MEFs after 72 h in suspension (Figure 4c). Similarly, there was no significant difference in cell death in EN- or K-Ras-transformed *ATG5*<sup>+/+</sup> versus *ATG5*<sup>-/-</sup> MEFs at 12 and 24 h of suspension, whereas at these time points there were significant differences in cell death in



**Figure 4** Autophagic flux is increased after cellular detachment, but loss of autophagy is insufficient to restore *anoikis* in EN- or K-Ras-transformed cells. (a) Western blot of LC3B-II levels in *AMPK*<sup>+/+</sup> versus *AMPK*<sup>-/-</sup> MEFs expressing EN or K-Ras versus MSCV vector alone after bafilomycin-A inhibition to assay for autophagic flux in monolayer versus 24-h suspension cultures.  $\beta$ -Actin is used as a loading control. (b) Densitometric quantification of the ratios of LC3B-II/ $\beta$ -actin immunoblot levels in EN- or K-Ras-transformed *AMPK*<sup>+/+</sup> versus *AMPK*<sup>-/-</sup> MEFs. (c) Caspase-3 activity in EN- or K-Ras-, or MSCV vector-alone-expressing *ATG5*<sup>+/+</sup> versus *ATG5*<sup>-/-</sup> MEFs after 72 h in suspension. All data are shown as mean  $\pm$  S.E.M. ( $n = 3$ ). The asterisk indicates statistical significance as determined by Student's *t*-test ( $P < 0.05$ )

transformed *AMPK*<sup>+/+</sup> versus *AMPK*<sup>-/-</sup> MEFs (Supplementary Figure S4). Therefore, defective macroautophagy is insufficient to restore *anoikis* to transformed cells at either early or prolonged time points, arguing against a major role for this process in AMPK-mediated *anoikis* resistance.

**Inhibition of protein synthesis reduces energy stress after cellular detachment and promotes *anoikis* resistance.** We next investigated whether the mTORC1-mediated inhibition of protein synthesis promotes *anoikis*

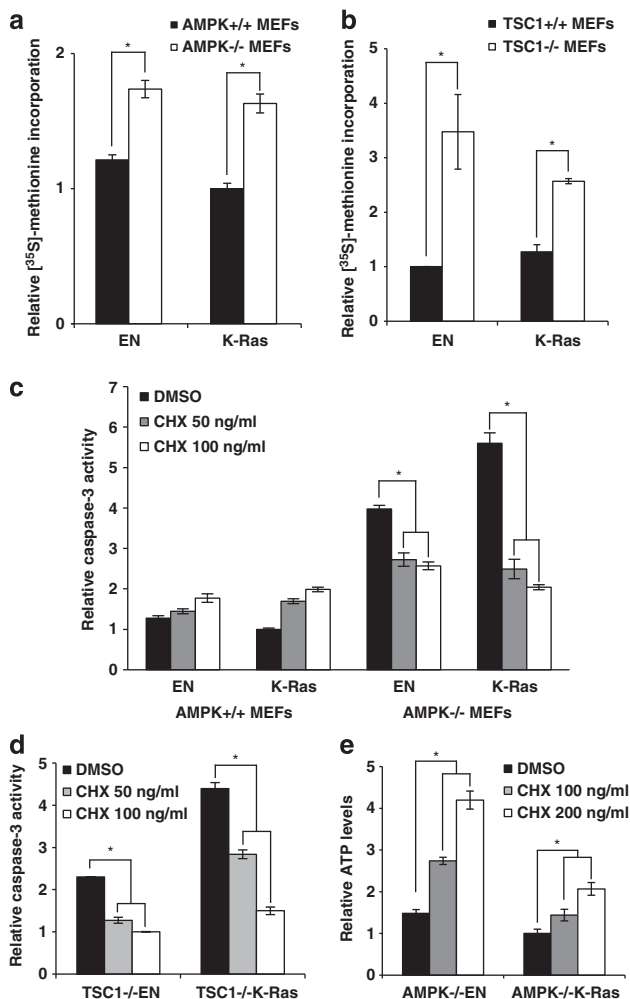
resistance in transformed cells, as protein synthesis is highly demanding bioenergetically.<sup>23</sup> First, using transformed *AMPK*<sup>-/-</sup> or *TSC1*<sup>-/-</sup> MEFs, we observed increased global protein synthesis rates in comparison with their *wt* counterparts (Figures 5a and b), suggesting that *anoikis* susceptibility is linked to their inability to suppress protein synthesis. To directly determine whether reduced protein synthesis promotes survival, *AMPK*<sup>-/-</sup>/EN and *AMPK*<sup>-/-</sup>/K-Ras MEFs were treated with cycloheximide (CHX) immediately after detachment. For these experiments we used considerably lower concentrations than for standard translation inhibition assays (i.e., 50–200 ng/ml versus 10–20 μg/ml) and in the range where its effects on

translation are linear and dose-dependent.<sup>24</sup> Low-dose CHX indeed partially restored survival in transformed *AMPK*<sup>-/-</sup> cells under detachment (Figure 5c and Supplementary Figure S3d). Interestingly, CHX had little effect on survival in transformed *AMPK*<sup>+/+</sup> MEFs under detachment, and in fact showed a trend toward reducing survival. This is consistent with its pro-survival effects being limited to cells that fail to suppress mTORC1 activity and protein synthesis. Similar effects were observed in transformed *TSC1*<sup>-/-</sup> MEFs (Figure 5d and Supplementary Figure S3h). Finally, as seen with rapamycin, CHX restored ATP levels in *AMPK*<sup>-/-</sup>/EN and *AMPK*<sup>-/-</sup>/K-Ras MEFs under detachment (Figure 5e). The ability of rapamycin and CHX to promote *anoikis* resistance was not through suppression of p53 translation (Supplementary Figure S5), as was reported previously in adherent cells under glucose deprivation.<sup>25</sup> Therefore *anoikis* resistance mediated by translation inhibition may be due in part to the mitigation of the metabolic defects occurring after loss of attachment. Overall, these data point to a model whereby detachment stress leads to AMPK-mediated mTORC1 inhibition, thus suppressing *anoikis* by preserving bioenergetic levels through a block in protein translation.

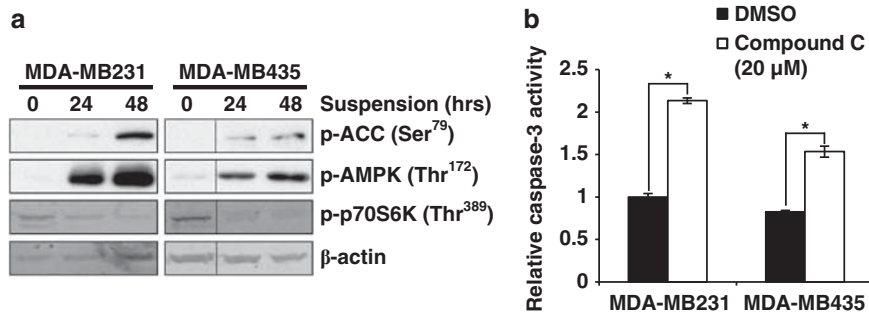
**AMPK activation contributes to *anoikis* resistance in breast carcinoma cell lines.** It is reported that fibroblasts are relatively more resistant to *anoikis* than epithelial cells.<sup>26</sup> We have observed that mesenchymal cell lines such as murine fibroblasts and human sarcoma cell lines demonstrate robust *anoikis* under detachment conditions when key *anoikis* suppressors such as IGF1R or ERBB4 are inhibited.<sup>8,27</sup> Nevertheless, we wished to extend our current findings to an epithelial cancer model. Using two metastatic breast carcinoma cell lines, MDA-MB231 and MDA-MB435, both demonstrated previously to be *anoikis*-resistant,<sup>28</sup> we analyzed the activity of the AMPK and mTOR pathways after detachment. Consistent with results in fibroblasts, both cell lines activate AMPK and suppress mTORC1 after detachment (Figure 6a). Furthermore, *anoikis* was restored after compound-C inhibition of AMPK activation under detachment conditions (Figure 6b). Therefore, our finding that AMPK activation contributes to *anoikis* resistance is applicable to transformed epithelial cells as well.

## Discussion

Cancer cells face diverse forms of stress during the metastatic process as they traverse harsh microenvironments and energy-depleted conditions. Many such stresses, including nutrient depletion and hypoxia, may be transient and cancer cells must rapidly respond to these conditions in order to survive. Various stress response pathways are critical for such survival, and reliance of cancer cells on these pathways has been termed ‘non-oncogene addiction’. Here we have shown that cellular detachment elicits a broad stress response in cancer cells, which is likely critical for *anoikis* resistance. In particular, we show that AMPK activation promotes survival in detached cells by mitigating reductions in ATP levels, and that this is linked to a block in mTORC1 signaling and global protein synthesis. Paradoxically,



**Figure 5** Inhibition of protein synthesis partially restores ATP levels and promotes anoikis resistance in cells with sustained mTORC1 activity after cellular detachment. (a and b) Assay of global protein synthesis rates as measured by levels of <sup>35</sup>S-methionine/cysteine incorporation into newly synthesized proteins in *AMPK*<sup>+/+</sup> or *AMPK*<sup>-/-</sup> versus *AMPK*<sup>-/-</sup> or *TSC1*<sup>-/-</sup> MEFs expressing EN or K-Ras 48 h after detachment. (c) Caspase-3 activity in *AMPK*<sup>+/+</sup> versus *AMPK*<sup>-/-</sup> EN- or K-Ras-transformed MEFs treated with low-dose CHX and assayed after 48 h in suspension. (d) Caspase-3 activity in CHX-treated *TSC1*<sup>-/-</sup> MEFs transformed with EN or K-Ras after 48 h in suspension. (e) ATP levels in CHX-treated *AMPK*<sup>-/-</sup> MEFs transformed with EN or K-Ras after 24 h in suspension. All data are shown as mean ± S.E.M. (n = 3). The asterisk indicates statistical significance as determined by Student's t-test (P < 0.05)



**Figure 6** *Anoikis*-resistant breast carcinoma cell lines activate AMPK and suppress mTORC1 after detachment, and AMPK inhibition partially restores *anoikis*. (a) Western blot demonstrating levels of p-ACC, p-AMPK, and p-p70S6K as readouts of AMPK and mTORC1 pathway activity in MDA-MB-231 and MDA-MB-435 breast cancer cell lines at the indicated time points after detachment.  $\beta$ -Actin is used as a loading control. (b) Caspase-3 activity assay of MDA-MB-231 and MDA-MB-435 cell lines treated with compound-C immediately after detachment and cultured in suspension for 48 h. All data are shown as mean  $\pm$  S.E.M. ( $n = 3$ ). The asterisk indicates statistical significance as determined by Student's *t*-test ( $P < 0.05$ )

mTORC1 inhibition relies on the expression of oncogenes such as activated K-Ras or EN to suppress mTORC1 activity after detachment. These oncogenes typically hyper-activate mTORC1 through sustained Akt activity during proliferative conditions.<sup>3</sup> Therefore, activation of this AMPK–mTORC1 cascade appears to be dominant over pro-growth and metabolically demanding pathways such as those driven by Akt, demonstrating the emphasis cancer cells place on stress response pathways to maintain survival during episodes of bioenergetic compromise.

Despite the functional diversity of AMPK- and mTOR-regulated pathways, their relative roles in *anoikis* resistance have not been well-studied. AMPK activation was previously demonstrated after detachment in breast epithelial cells, and was associated with induction of autophagy.<sup>10</sup> However, the relative contribution of AMPK activation to survival and/or autophagy was not determined. Overexpression of mTOR in non-transformed *wt* fibroblasts promoted *anoikis* resistance, and mTOR knockdown in MEFs deficient in all retinoblastoma-family members restored *anoikis*.<sup>29</sup> However, whether mTOR overexpression correlates with gain-of-function of downstream pathways, particularly in relation to mTORC1 *versus* mTORC2 complex function, was not determined. Interestingly, in thyroid epithelial cells, inhibition of protein synthesis using low-dose CHX also promoted survival in suspension but showed no effect on the survival of monolayer cells,<sup>30</sup> supporting our finding that translation inhibition promotes *anoikis* resistance.

Recently, a pro-survival role for macroautophagy in anchorage-independent growth was reported.<sup>31</sup> Consistent with our data, these authors demonstrated that detachment of H-Ras(V12)-transformed cells led to the activation of autophagy in association with mTORC1 inhibition. However, *ATG5* deficiency in H-Ras(V12)-transformed MEFs led to increased apoptosis under anchorage-independent conditions, contrary to our findings in which EN- and K-Ras(V12)-transformed MEFs, whether deficient or *wt* for *ATG5*, were able to suppress *anoikis*. Several recent studies demonstrated a key role for macroautophagy in proliferation and metabolism during Ras-mediated transformation.<sup>32,33</sup> In these studies, whereas macroautophagy was critically important to maintain a metabolic phenotype permissive for sustained proliferation during transformation,

a function for autophagy in suppressing *anoikis* after cellular detachment was not required for Ras transformation. In fact, direct suppression of apoptosis through Bcl-2 overexpression was insufficient to restore prolonged anchorage-independent growth in Ras-transformed, autophagy-deficient cells.<sup>31</sup> Our data suggest that AMPK is important for both prolonged anchorage-independent growth, as demonstrated previously,<sup>34</sup> and for survival at early time periods following detachment, as shown in Figures 2c and 4c, and Supplementary Figure S4. Therefore, whereas autophagy is required for certain essential components of Ras-mediated transformation such as proliferation and altered metabolism, *anoikis* resistance appears to represent a divergent oncogenic property potentially dependent on other processes such as activation of the AMPK pathway.

Consistent with our results, a pro-survival role for AMPK activation and mTORC1 inhibition has been documented under other stress conditions. AMPK activation promotes survival during nutrient deprivation, which appears to be dependent on an AMPK-mediated cell-cycle checkpoint.<sup>35</sup> Under starvation and DNA damage, constitutive mTORC1 activity in the context of tuberous sclerosis complex (TSC) deficiency compromises survival through modulation of p53 levels.<sup>25</sup> Inhibition of mTORC1 also promotes survival in TSC-deficient cells during glucose starvation by maintaining energy levels.<sup>36</sup> Interestingly, TSC2 overexpression has been associated with increased tumor invasion and metastasis,<sup>37</sup> contrary to its well-studied tumor-suppressive role. 4E-BP levels also correlate with metastatic potential, allowing cells to better tolerate hypoxic stress, possibly through its ability to promote a switch to the cap-independent translation of pro-survival factors such as VEGF-A and Bcl-2.<sup>38,39</sup>

In summary, our data indicate that cellular detachment generates significant energy stress, and that bioenergetic homeostasis after detachment is necessary for survival. We demonstrate a novel *anoikis* resistance pathway mediated by an AMPK–mTORC1 axis that suppresses protein synthesis. This axis appears to be specifically important for oncogene-transformed cells. The AMPK pathway is broadly considered to be tumor-suppressive in nature, and pharmacologic activation of AMPK or inhibition of mTOR is currently under investigation as components of cancer therapy. However,



consideration of how these pathways may alter survival and potentially metastatic potential through their ability to suppress *anoikis* will be important in the development of cancer therapeutics and in our understanding of cancer progression.

## Materials and Methods

**Cell culture and DNA transfection.** Early-passage NIH3T3 were obtained from ATCC (Rockville, MD, USA). *AMPK*<sup>+/+</sup> and *AMPK*<sup>-/-</sup> MEFs were obtained as described previously.<sup>34</sup> *TSC1*<sup>+/+</sup> and *TSC1*<sup>-/-</sup> MEFs were provided by David Kwiatkowski (Harvard Medical School, Boston, MA, USA). *4E-BP1*/Ras *wt* and DKO MEFs were provided by Nahum Sonenberg (McGill University, Montreal, QC, Canada). *ATG5*<sup>+/+</sup> and *ATG5*<sup>-/-</sup> MEFs were provided by Noboru Mizushima (National Institute for Basic Biology, Okazaki, Japan). *IGF1R*<sup>-/-</sup> MEFs (R<sup>-</sup>) and *IGF1R*<sup>-/-</sup> MEFs re-expressing *IGF1R* (R<sup>+</sup>) were provided by Renato Baserga (Kimmel Cancer Centre, Philadelphia, PA, USA). The retroviral expression vectors pMSCVpuro-ETV6-NTRK3 and pMSCVpuro-ETV6-NTRK3myr were used for generation of stable cell lines as described previously.<sup>8</sup> The pMSCVpuro-K-Ras(V12) vector was constructed by subcloning the *Bam*HI–*Sal*I fragment from the pBabe-puro-K-Ras(V12) vector obtained from Addgene (courtesy of William Hahn) containing the K-Ras(V12) open reading frame into the *Bgl*II–*Xho*I sites of pMSCVpuro. The pMSCVneo-DN-AMPK vector was constructed by subcloning the *Eco*RI fragment from pBabe-GFP-DN AMPK (gift from Nissim Hay) into the pMSCVneo vector. Infections of the NIH3T3 cells and MEFs were performed by using retrovirus generated after introduction of the retroviral vectors into PhoenixA cells. For suspension cultures, cells at sub-confluence were detached by trypsinization and diluted to a concentration of 250 000 cells/ml, plated on polyHEMA-coated 10-cm dishes or 6- to 12-well plates, or pre-coated 96-well plates (Non-Binding Surface coated microplates; Corning, Lowell, MA, USA), and incubated for the indicated time periods. Chemical inhibitors were added to suspension cultures immediately after detachment and plating onto polyHEMA-coated plates to determine their effects on the early stages of the *anoikis* response.

**Antibodies and chemicals.** The anti-p-ACC (S79), anti-ACC, anti-p-Raptor (S792), anti-p-AMPK $\alpha$  (T172), anti-cleaved caspase-3, anti-p-p70 S6 kinase (T389), anti-4E-BP1, anti-p-Akt (S473), anti-p-MEK1/2 (S217/221), anti-GAPDH, and anti-LC3B antibodies were purchased from Cell Signaling (Danvers, MA, USA). The anti-TrkC, to detect ETV6-NTRK3 expression, and anti- $\beta$ -actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The anti-K-Ras antibody was purchased from Calbiochem/EMD Biosciences (Darmstadt, Germany). Compound-C was obtained from Calbiochem/EMD Biosciences. Rapamycin was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). CHX was obtained from Sigma-Aldrich (Oakville, ON, Canada). Bafilomycin-A was obtained from A.G. Scientific (San Diego, CA, USA).

**Caspase-3 activity assay.** Cells were lysed in caspase-3 lysis buffer (10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.2% CHAPS) and assayed for protein concentration by the D<sub>C</sub> protein assay (Bio-Rad, Mississauga, ON, Canada). Lysates were mixed at a 1 : 1 ratio with caspase-3 reaction buffer (40 mM PIPES (pH 7.2), 200 mM NaCl, 20% sucrose, 0.2% CHAPS, 20 mM DTT); caspase-3 substrate was added to a concentration of 0.5  $\mu$ M (Caspase-3 Substrate IX fluorogenic; Calbiochem/EMD Biosciences), and incubated for 1 h at 37°C. Fluorescence intensity was measured and normalized to protein concentration.

**Annexin-V/7-AAD FACS assay.** Transformed and non-transformed *AMPK* MEFs were cultured in suspension for 48 h, harvested by centrifugation, washed, and trypsinized for 10 min. These dispersed cells were washed, centrifuged, and filtered (0.45  $\mu$ m) to obtain a single-cell suspension. Annexin-V-FITC and 7-AAD were added with the cells in binding buffer according to the manufacturer's protocol (BD Pharmingen, Mississauga, ON, Canada); incubated at room temperature for 15 min; and subjected to FACS analysis.

**Cell survival and cytotoxicity assays.** Cell survival was measured by using the AlamarBlue assay (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Briefly, cells were detached and cultured for 48 h in suspension on a 96-well coated plate. The AlamarBlue reagent was added at a 1 : 10 ratio, incubated for 4 h at 37°C, and absorbance was read and corrected for background. The LDH release Cytotoxicity Detection Kit (Roche, Indianapolis, IN, USA) was used according to the manufacturer's instructions. Briefly, cells were

detached and cultured for 48 h in suspension on a six-well coated plate. Cleared supernatant media was collected; combined with the LDH assay reaction mixture; incubated for 0.5 h; and absorbance was read, corrected for background, and normalized to the total cell count. The Cytotox-Glo cytotoxicity assay (Promega, Madison, WI, USA) was performed according to the manufacturer's protocol. Briefly, cells were detached and cultured for 24 h in suspension on a 96-well coated plate. The assay reagent was added at room temperature, incubated for 15 min, and total luminescence was measured, corresponding to the protease levels released by dead cells. This value was normalized to total cell protease activity after re-incubation for 15 min with additional assay reagent combined with digitonin for complete cell lysis, as provided in the assay kit.

**ATP assay.** Cells were lysed in ATP assay lysis buffer (10 mM Tris (pH 7.5), 40 mM NaCl, 1% Triton X-100, 20 mM EDTA) supplemented with a protease inhibitor cocktail (Complete Mini tablets; Roche) and assayed for protein concentration. Lysates were mixed at a 1 : 1 ratio with the ATP reaction buffer in the ATP Bioluminescent Assay kit (Sigma-Aldrich) at room temperature and luminescence was measured immediately. The luminescence values were normalized to protein concentration.

**Total protein synthesis assay.** Cells were cultured in suspension conditions and pulsed with 10  $\mu$ Ci [<sup>35</sup>S]-methionine/cysteine mix (EasyTag EXPRESS Protein Labeling Mix; Perkin Elmer, Woodbridge, ON, Canada) for 30 min. Cells were washed then lysed by using buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40, and protease inhibitors (Roche). Using 10  $\mu$ g of lysate, total protein was precipitated by using 10% trichloroacetic acid (TCA), vacuum-filtered using glass microfiber filters (GF/C; Whatman, Piscataway, NJ, USA), washed with TCA and ethanol, and total [<sup>35</sup>S] activity was measured to reflect methionine incorporation and global protein synthesis.

**Gene expression profiling.** Biotin-labeled cRNA was prepared from total RNA and hybridized to Affymetrix GeneChip Mouse 430A Expression Arrays according to the manufacturer's protocol (Affymetrix Inc., Santa Clara, CA, USA). Array data were normalized by using the Robust Multi-Array Average module in the Genetrix software package (Epicenter Software, Pasadena, CA, USA), which was used for subsequent gene expression analysis. The normalized expression values were log-transformed and genes were selected as differentially expressed based on at least two-fold mean difference in expression between the monolayer (R + EN/R-EN/R-ENmyr) and suspension (SPHR + EN/SPHR-EN/SPHR-ENmyr) groups, each cultured in their respective conditions for 24 h, and *t*-tests were used to determine significance (*P* < 0.05). Functional annotation was performed by using the DAVID online tool for overrepresentation analysis of functional gene categories and GO terms. GSEA between the monolayer and suspension phenotypes was performed as described previously,<sup>40</sup> using the Broad Institute software (v 2.07), with the analysis performed by using permutations of gene sets (1000) and Signal2Noise metric for ranking of genes.

## Conflict of Interest

The authors declare no conflict of interest.

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