tRNA biogenesis and specific Aminoacyl-tRNA Synthetases regulate senescence stability under the control of mTOR

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Running title: tRNA deregulation during senescence

Keywords: Chemoresistance senescence, mTOR, tRNA, ER stress.

ABSTRACT

Senescence normally prevents the propagation of abnormal cells but is also associated with cancer progression and chemotherapy resistance. These contradictory effects are related to the stability of epigenetic marks and to the senescence-associated secretory phenotype (SASP). The SASP reinforces the proliferative arrest but also induces tumor growth and inflammation during aging. Senescence is therefore much more heterogeneous than initially thought. How this response varies is not really understood.

Using experimental models of senescence escape, we now described that the deregulation of tRNA biogenesis affects the stability of this suppression, leading to chemotherapy resistance. Proteomic analyses showed that several aminoacyl-tRNA synthetases were down-regulated in senescent cells. tRNA transcription was also inhibited as a consequence of a reduced DNA binding of the type III RNA polymerase. Reducing RNA Pol III activity by BRF1 depletion maintained senescence and blocked cell persistence. Results showed that the YARS1 and LARS1 aminoacyl-tRNA synthetases were necessary for cell emergence and that their corresponding tRNA-Leu-CAA and tRNA-Tyr-GTA were up-regulated in persistent cells. On the contrary, the CARS1 ligase had no effect on persistence and the expression of the corresponding tRNA-Cys was not modified. Results also showed that these tRNAs were regulated by mTOR and that this abnormal tRNA biogenesis induced an ER stress which was resolved by the kinase during chemotherapy escape.

Overall, these findings highlight a new regulation of tRNA biology during senescence and suggest that specific tRNAs and ligases contribute to the strength and heterogeneity of this suppression and to chemotherapy resistance.

INTRODUCTION

Senescence induces a definitive proliferative arrest in response to telomere shortening, oncogenes or chemotherapy (1,2). This tumor suppression is most of the time induced by DNA damage and activation of the p53-p21 and p16-Rb pathways. A definitive proliferative arrest is then maintained by the Rb-mediated compaction of proliferative genes within heterochromatin foci or SAHFs (Senescence Associated Heterochromatin Foci) (3). Senescence is also characterized by an increased permeability of the nuclear envelope which allows leakage of chromatin fragments within the cytoplasm. These abnormal fragments are detected by the cGAS-STING DNA sensing pathway which then activates NF-kB and induces the production of a specific secretome known as the SASP (Senescence-Associated Secretory Phenotype). Mainly composed of cytokines and chemokines, this secretome maintains senescence and attracts immune cells which then eliminate the senescent populations (4-7). Thus, through the up-regulation of the p53 and Rb pathways and the activation of immune responses, senescence prevents the propagation of abnormal cells.

Although initially described as a definitive proliferative arrest, we and others have recently shown that some cells can escape senescence, indicating that distinct stages of light and deep senescence should be distinguished (2,7-9). This heterogeneity can be explained by a variable expression of p16 which is necessary for senescence maintenance. The stability of senescence also implies that the compaction of proliferative genes is maintained within the SAHFs. Recent results have reported that H3K9Me3 repressive marks can be removed by the JMJD2C and LSD1 demethylases and that this induces senescence escape (10). The HIRA histone chaperone also plays a key role in the deposition of the histones H3.3 and H4 into the chromatin. Its down-regulation allows senescence escape, indicating again that the stability of epigenetic marks plays a key role in the maintenance of the suppressive arrest. The dynamic nature of senescence is also explained by the variability of the SASP. Initially described as beneficial, several studies have reported that its composition varies and that this secretome can also enhance inflammation or tumor progression (11-13). The reason for this variability is not really understood. Cancer cell lines also represent an interesting model of an incomplete senescence response. We and others have shown that this suppression functions as an adaptive mechanism in response to chemotherapy-induced -senescence (CIS) (7,9). We have described that cancer cells can escape senescence and emerge as more transformed cells that resist anoikis and are more invasive (14-17). In addition, we have also shown that cells having an incomplete senescence response are characterized by a reduced expression of CD47 (17). As previously proposed (18,19), this indicates that senescent populations are heterogeneous and can be identified by cell surface receptors.

Taken together, these studies indicate that senescence is much more dynamic than initially thought and that a better characterization of these arrested cells is necessary. At the single cell level, any modification of epigenetic marks, of the SASP composition or any variability of the oncogenic

background will generate heterogeneous populations. In this study, we pursued our experiments with the aim of characterizing the signaling pathways involved in the maintenance of senescence. We describe in this work that mTOR activates tRNAs synthesis during senescence escape. During the initial steps of this suppressive arrest, the transcription of the type III RNA polymerase is down-regulated and tRNA synthesis is then reactivated during senescence escape. Depending on the experimental model, results showed that specific tRNAs were reactivated, such as the $tRNA^{Leu}$-CAA and $tRNA^{Tyr}$-GTA tRNAs in emergent colorectal cells or breast organoids. In addition, specific aminoacyl-tRNA synthetases (ARS) such as the Leucyl-and Tyrosyl-tRNA ligases were necessary for senescence escape. Our results also indicate that this deregulation of tRNA synthesis led to the activation of the unfolded protein response (UPR) and that this tRNA-mediated ER stress is resolved by mTOR to allow senescence escape.

Altogether, these results indicate that specific pools of tRNAs or ARSs regulate the outcome of this suppressive response and of chemotherapy. We propose that different types of senescence, replicative, oncogenic or mediated by chemotherapy might lead to the expression of different pools of tRNA and ARSs. This could partly explain the variability of the SASP and the deleterious effects of senescence in response to treatment.

RESULTS

mTOR inhibition prevents CIS escape

In breast and colorectal cell lines that do not express p16INK4, we have previously reported that p21 maintains senescence and that its down-regulation allows CIS escape (17). To confirm this observation, senescence was induced with sn38 for 96hr, cells were then transfected with control siRNA or siRNA directed against p21 and emergence was evaluated after 10 days (Figure 1A). Western blot analysis confirmed the down-regulation of the cell cycle inhibitor and showed a concomitant up-regulation of cyclin A and of phospho-Rb (Figure 1B). As we have shown (17), this led to a significant increase in CIS escape (Figure 1C). This effect was observed in LS174T colorectal cells and in MCF7 breast cells (Supplementary Figure 1A). We then used mass spectrometry analysis to understand how p21 maintains senescence besides its well-known function as a cdk inhibitor. To this end, this protein was inactivated in senescent cells, extracts were collected after 48 hr and analyzed by SWATH−MS approaches as we recently described (20). GSEA analysis indicated that Myc and E2Fs proliferative pathways were reactivated as expected. Interestingly, a significant up-regulation of mTOR signaling was also detected (Figure 1D). We focused on this kinase since it plays a key role during senescence (21-25). Western blot analysis confirmed that mTOR was up-regulated in LS174T and MCF7 cells following p21 inhibition, as shown by the phosphorylation of the S6 ribosomal protein, one of its main target (Figure 1E). We then asked if mTOR was involved in CIS escape by treating the cells at the beginning of emergence with torin-1 and rapamycin, two common drugs used to inactivate this kinase. These two inhibitors significantly blocked CIS escape following p21 inactivation (Figure 1F). Emergent cells were more sensitive to mTOR inhibition than parental, untreated cells (Supplementary Figure 1B). In addition, Rb phosphorylation or cyclin A expression were not inhibited by the two drugs (Figure 1G). We then determined if mTOR was also involved in spontaneous CIS escape, in the absence of p21 manipulation. Western blot analysis indicated that the kinase was activated at the early steps of emergence, two days after serum release (Figure 1H). In this condition, torin-1 or rapamycin inhibited its activity and significantly blocked CIS escape, both in LS174T and MCF7 cells (Figure 1H and I).

Altogether these observations indicate mTOR is necessary for CIS escape, either during spontaneous emergence or following p21 inactivation.

Activating ER stress prevents CIS escape

Interestingly, the mass spectrometry analysis also detected a significant deregulation of the Unfolded Protein Response (UPR) following p21 inactivation (Figure 2A). We and others have recently shown that the SASP induces tumor progression and senescence escape (11,17). In addition, this abnormal secretome induces protein misfolding and ER stress (26,27). This suggested that the protein stress generated during senescence has to be resolved to allow cell emergence. Indeed, RT-QPCR analysis indicated that several UPR regulators were significantly inhibited during senescence escape (Figure 2B). In light of these observations, we determined if increasing ER stress could prevent cell emergence. To this end, we used tunicamycin or thapsigargin, two well known inducers of the UPR pathway. When added on senescent cells, these two drugs significantly blocked CIS escape, both in L174T and MCF7 (Figure 2C and supplementary Figure 1C). As expected, they also induced a significant up-regulation of Bip and CHOP, two main regulators of ER stress signaling. To confirm this observation, we used siRNA to down-regulate the CHOP

transcription factor at the beginning of emergence since this protein is one of the general UPR regulator (see supplementary Figure 1D for the verification). Results presented Figure 2D show that CHOP inhibition significantly reduced CIS escape, both in LS174T and MCF7 cells. Finally, we then asked if this ER stress could be detected during emergence. We observed an increased Bip and CHOP expression in emergent MCF7 cells, but this was not the case in LS174T cells (Figure 2E).

Together, these results indicate that increasing UPR stress prevents CIS escape, suggesting that emergent cells are able to reduce these toxic effects. Interestingly, LS174T cells and MCF7 cells behave differently, indicating that the UPR is either not up-regulated in emergent colorectal cells or that these cells have a better efficiency to resolve the ER stress generated during senescence escape.

mTOR facilitates CIS escape in the presence of a protein stress

Since mTOR allows cell emergence, we then asked if this kinase reduces the UPR pathway. When the kinase was inhibited following senescence induction, a significant increase of Bip expression was detected in MCF7 cells. This effect was less evident in LS174T cells, again indicating a difference between the two cell lines (Figure 3A). We then determined if mTOR allowed CIS escape in the presence of an increased protein stress. We first down-regulated CHOP by siRNA in senescent cells and then induced emergence in the presence of torin-1 or rapamycin (note that the drugs were added after 24 hr of siRNA treatment, which is different from Figure 1I). Results showed that the combined inhibition of mTOR and CHOP led to a significant reduction of CIS escape, both in LS174T and MCF7 cells (Figure 3B). To confirm this observation, we then activated mTOR using a lentivirus encoding an shRNA directed against TSC2. As an inhibitor of the Reb GTPase, TSC2 is one of the main repressors of the kinase (28). Cells were infected after CIS induction, and senescent cells were treated with tunicamycin to increase the UPR stress and block emergence as described Figure 2C. Results showed that mTOR activation allowed CIS escape despite the increased protein stress generated by the drug. Interestingly, this effect was observed in MCF7 cells but also in LS174T cells (Figure 3C-D, left). As expected, the inactivation of TSC2 in MCF7 cells increased senescence escape. Interestingly, no effect was observed on the emergence of colorectal cells, indicating that mTOR becomes necessary in this cell line when the ER stress is too high (Figure 3 C-D, right).

Altogether these observations indicate that mTOR favors CIS escape when the ER stress is increased.

Regulation of tRNA synthesis by mTOR and during senescence

We then tried to understand the link between CIS escape, mTOR and the ER stress. In LS174T cells, we noticed that rapamycin and torin-1 always reduced the total RNA concentration during senescence escape. Conversely, when TSC2 was inactivated in MCF7 cells, this led to an increased RNA concentration (Supplementary Figure 2A). Since it has recently been demonstrated that specific tRNAs display unexpected functions during cancer progression and that some of these are regulated by mTOR (29), we then determined if tRNA expression was modified by torin-1 or rapamycin. Results presented in Figure 4A indicate that the expression of several tRNAs such as $tRNA^{Leu}$-CAA and $tRNA^{Tyr}$-GTA was reduced. Conversely, when mTOR was activated by the depletion of TSC2 in MCF7 cells, we observed an up-regulation of the $tRNA^{Tyr}$-GTA, of the $tRNA^{Leu}$-CAA and to a lesser extent of the other tRNAs. (Figure 4B). Results also showed that mTOR inhibition reduced the expression of the 5S rRNA but did not affect the pre45S and 18S rRNAs level in LS174T cells. Since these ribosomal RNAs are regulated by the type I RNA polymerase, this suggests that RNA Pol III activity is a main target in these experimental conditions.

In light of these results, we then determined if tRNA synthesis was affected during senescence. RT-QPCR results presented in Figure 4C indicate that the expression of tRNAs was down-regulated in senescent cells, both in LS174T and MCF7 cells. In contrast, the expression of the 18S and pre-45S ribosomal RNAs was not significantly modified. We then used ChIP experiments to analyze the recruitment of the type III RNA polymerase to the indicated tRNA genes (Figure 4D). RNA Pol III was detected on these promoters in growing LS174T cells but its binding was significantly reduced in senescent cells. To extend this observation, we performed mass spectrometry analysis of LS174T senescent cells. Results presented in Figure 4E indicated that a significant number of tRNA ligases were down-regulated during senescence. Note however that some were up-regulated, indicating that this suppressive arrest did not induce a general inactivation of tRNA signaling pathways.

At least for the tRNAs investigated, these results indicate that tRNA synthesis is down-regulated during senescence but that some ligases remain unaffected or up-regulated. This effect is therefore not related to a general down-regulation of the tRNA pathways. Given the complexity of

tRNA transcription, it remains to be determined if all tRNAs are affected to the same extent.

Reactivation of tRNA transcription during CIS escape

We then determined if the expression of the tRNAs was modulated during senescence escape. RT-QPCR results presented Figure 5A indicate that a significant increase of the $tRNA^{Leu}$-CAA and $tRNA^{Tyr}$-GTA tRNAs was observed in emergent LS174T cells. The same effect was observed in MCF7 cells where several tRNAs were reactivated. It is important to note that this did not correspond to a general increase of tRNA transcription but to a specific modulation. Several tRNAs were not up-regulated in emergent LS174T cells and the $tRNA^{Cys}$ was not significantly modified in the breast cancer cell line. In both cases, the expression of the 45S and 18S rRNAs was not modified. This suggested to us that a direct regulation of tRNA transcription might be sufficient to modify the senescence response. The type III RNA polymerase is regulated by the BRF1 and MAF1 proteins. Whereas BRF1 is necessary for RNA Pol III activity, MAF1 functions as a repressor by binding to BRF1. This transcriptional repression is controlled by mTOR which phosphorylates and inhibits MAF1 (29-31). To determine if there was a link between RNA Pol III transcription and senescence escape, we first inactivated MAF1 by RNA interference. As expected, RT-QPCR experiments indicated that MAF1 down-regulation led to an increased expression of tRNA expression, both in LS174T and MCF7 cells (Figure 5B, Supplementary Figure 2C). This led to a weak increase in senescence escape in LS174T cells but no significant effect was observed in the breast cell line (Figure 5C, left). At least for the tRNAs tested, a general increase in expression was therefore not sufficient to induce cell emergence. In contrast, when BRF1 was down-regulated by siRNA, a significant inhibition of senescence escape was observed in the two cell lines (Figure 5C right). After 7 days of emergence and cell fixation, a microscopic examination allowed the visualization of emergent, dividing, white clones in the middle of blue senescent cells identified by SA-ß-galactosidase staining. These dividing, white clones were completely absent when BRF1 was inactivated, indicating that this inhibition allowed the maintenance of senescence (Supplementary Figure 2B). As expected, BRF1 down-regulation led to a reduced expression of tRNA expression in MCF7 cells (Supplementary Figure 2D). In LS174T cells, the $tRNA^{Cys}$ and $tRNA^{His}$ were less affected (Figure 5B, right), suggesting that the inhibition of emergence may not be due to a general inhibition of tRNA synthesis in these cells. In addition, we observed that parental, untreated LS174T cells were less sensitive to BRF1 inhibition than MCF7 cells (Supplementary Figure 2E). To extend this observation, we also down-regulated by siRNA the expression of the aminoacyl-tRNA synthetases (ARS) involved in the tRNA ligation of the leucine (LARS), tyrosine (YARS) and cystein (CARS) amino acids (see the validation in Supplementary Figure 3). Results presented in Figure 5D indicate that LARS and YARS inactivation reduced senescence escape, both in LS174T and MCF7 cells. Importantly, no effect was seen when the CARS ligase was inhibited.

Finally, we determined if a deregulation of BRF1 and tRNA transcription led to an increased ER stress. Western blot analysis indicated that the inactivation of this protein led to a significant induction of the Bip sensor in MCF7 cells (Figure 5E). Note that this was not observed in LS174T cells, further indicating that these cells may tolerate a higher level of protein stress.

Altogether these observations indicate that tRNA transcription is down-regulated in senescent cells and that BRF1 and specific aminoacyl-tRNA synthetases such as LARS and YARS are necessary during emergence. At least in LS174T cells, the RNA^{Leu}-CAA and $tRNA^{Tyr}$-GTA tRNAs are reactivated, indicating that senescence escape can rely on specific tRNAs and ARS.

Up-regulation of tRNA transcription in breast organoids that escape chemotherapy

To corroborate that tRNA transcription is also specifically reactivated in primary cancer cells, we used organoids derived from freshly excised human breast cancer specimens (see a representative image in Figure 6A, (32)). PDOs isolated from two different patients were treated with doxorubicin and pilot experiments indicated that a proliferative arrest was induced after two sequential drug treatments for 96 hr. As these cells grew in three dimensions, the SA-ß-galactosidase staining was constitutively positive within the inner part of the spheroid, precluding the use of this marker. RT-QPCR experiments showed that proliferative genes such as mcm2 or cdc25A were down-regulated, together with an up-regulation of IL-8 as an illustrative member of the SASP (Figure 6B). P21 expression was also increased in the two PDOs but this was not the case of p16, probably because this gene was inactivated during the initial steps of cell transformation. After chemotherapy treatment, we observed that some PDOs restart proliferation and this was illustrated after 10 days by the reactivation of mcm2 and cdc25A and the down-regulation of p21 (Figure 6C). To test the influence of senescent cells, we used ABT-263 which is widely recognized as a senolytic compound. We first validated its effect on MCF7

cells. Results presented in Figure 6D show that this drug weakly modified the proliferation of parental cells whereas it completely blocked the senescence escape of doxorubicin-treated cells. When used on PDOs, ABT-263 reduced to some extent the viability of untreated spheroids, probably because senescent cells are present within the inner mass of these spheroids (Figure 6E). Interestingly, chemotherapy escape was reduced by the use of ABT-263, indicating that senescent cells present within the PDOs participate in the emergence (Figure 6E). Finally, we evaluated tRNA expression after 10 days of emergence. Results presented in Figure 6F indicate that the $tRNA^{Leu}$-CAA and $tRNA^{Tyr}$-GTA were up-regulated in the two different PDOs whereas this was not the case for the other tRNAs and ribosomal RNAs tested.

Altogether these observations indicate that primary cancer cells grown as PDO can also escape chemotherapy treatment. Given the limited material available, we did not have enough evidence to really conclude that these cells escape senescence. Nevertheless, in this condition of treatment escape, we also observed an up-regulation of specific tRNAs when PDO restarted their proliferation.

DISCUSSION

Several studies have reported that senescence is much more dynamic than initially expected. How this heterogeneity is regulated remains largely unknown. It can be related to specific transcriptional activities, to the maintenance of epigenetic marks, to the variability of the SASP or to a specific oncogenic background in the case of cancer cells. Single-cell variability is expected to generate subpopulations of cells that enter distinct states of light or deep senescence and as a result some cells will escape chemotherapy more easily. In this study, we extend these observations and propose that the strength of CIS is related to the expression of specific tRNAs and aminoacyl-tRNA synthetases.

It is known that perturbations of ribosome biogenesis induce suppressive pathways. Ribosomal proteins such as L11 can interact with hdm2 to activate p53 and induce cell cycle arrest (33). Ribosomes are also controlled in a p53-independent manner since the RPS14 protein can interact with cdk4 to prevent its activity. This inhibits Rb phosphorylation and induces senescence as a response to an abnormal ribosome biogenesis (34). Our results indicate that tRNA transcription is inhibited during the initial step of the suppressive arrest but that emergent cells reactivate this activity to allow senescence escape. This effect is specific and not related to a general activation of RNA Pol III since only specific tRNAs are up-regulated during emergence. Of the aminoacyl-tRNA synthetases tested, only the LARS and YARS tRNA ligases allowed senescence escape. The CARS ligase did not affect emergence and the expression of the corresponding $tRNA^{Cys}$ was not modified. Given the complexity of tRNA biology, it remains to be determined on a larger scale if other tRNAs and ARSs are involved in cell emergence. We speculate that this will be the case and that different pools of tRNAs and ligases will regulate senescence pathways. A specific expression could explain the heterogeneity of this response and the ability of cells to remain definitely arrested or conversely to restart proliferation.

Further experiments are necessary to determine how tRNAs and ARSs regulate CIS and cell emergence. This may be related to protein synthesis but it should be noted that some aminoacyl-tRNA synthetases have adopted new functions during evolution, beyond protein synthesis. For instance, the leucyl-tRNA synthetase can induce mTOR activity upon leucine stimulation (35). The Trp-tRNA synthetase can interact with DNA-PK and PARP to activate p53 (36). It has also been recently reported that the seryl tRNA synthetase interacts with the POT1 member of the telomeric shelterin complex and that this accelerates replicative senescence (37). Thus, we can speculate that the LARS and YARS tRNA ligases have acquired new functions that somehow allow senescence escape.

New results have also described unexpected functions for tRNAs (38,39). Recent findings show that $tRNA^{Glu}$ and $tRNA^{Arg}$ are over-expressed in metastatic cells and that their inactivation blocks *in vivo* metastasis (40). These tRNAs induce a profound change in the proteome, with enrichment of proteins containing the GAA and GAG codons in their genes. This effect is specific, as it is not seen with other tRNAs. Santos et al. have recently reported that mutant tRNAs that mis-incorporate Serine instead of Alanine induce cell transformation (41). It has been proposed that a change in the pool of available tRNAs can lead to statistical errors in tRNA load within the ribosomal subunits (38,39). This generates a random proteome known as a statistical proteome, which represents mutated proteins or proteins with a novel sequence not completely coded for by the genome (42,43). It will be interesting to determine if the variability of the SASP might be explained by the utilization of different tRNA pools. Although this remains to be demonstrated, it leads to the hypothesis that subpopulations of senescent cells might express specific tRNAs (or tRNA ligases) and that this will allow the expression of secretomes presenting distinct, specific inflammatory activities.

Further experiments are therefore needed to determine if different pools of tRNAs and aminoacyl-tRNA synthetases are expressed in

response to oncogenic insults, chemotherapy or during aging. We speculate that each type of suppressive arrest might lead to the expression of specific tRNAs and ARSs and that this might explain the specificity of these responses. In addition to epigenetic and transcriptional regulations, we therefore propose that the heterogeneity of tRNAs and ligases expression also leads to distinct states of light or deep senescence. This observation provides a rationale to further study the different facets of senescence responses and their links with chemotherapy resistance.

EXPERIMENTAL PROCEDURES

See also the supplementary file

Cell lines, senescence induction and generation of persistent cells
LS174T and MCF7 cell lines were obtained from the American Type Culture Collection. Cell lines were authenticated by STR profiling and were regularly tested to exclude mycoplasma contamination. To induce senescence, cell lines were treated for 96 hr in RPMI medium containing 3% of SVF with Sn38 (5 ng/ml, LS174T) or Doxorubicin (25 ng/ml, MCF7). To promote senescence escape cells were washed with PBS and stimulated with fresh medium containing 10% SVF for 7 (RT-qPCR analysis, Western Blot, SA-β galactosidase staining) or 10 days (evaluation of emerging clone number).

Treatments:
Cells were treated with the following drugs:
Torin-1 (Cell Signaling, 14379): 15 nM, Rapamycin (Santa-Cruz, sc-3504): 5 nM, Tunicamycin (Santa-Cruz, sc-3506): 0.1 to 8 µg/ml, Thapsigargin (Santa-Cruz, sc-24017): 10 to 50 nM

SiRNA transfection
Cells were transfected with 50 nM of small interfering RNA against p21(CDKN1A) (ON-TARGET plus Human CDKN1A (1026) Dharmacon, L-00341-00-0005), CHOP (DDIT3) : (ON-TARGET plus Human DDIT3, Dharmacon, L-004819-00-0005), Maf1 (ON-TARGET plus Human MAF1, Dharmacon, L-018603-01-0005), BRF1 (ON-TARGET plus human BRF1, Dharmacon, L-017422-00-0005), LARS (ON-TARGETplus Human LARS siRNA, L-010171-00-0005), YARS (ON-TARGETplus Human YARS siRNA, L-011498-00-0005), CARS (ON-TARGETplus Human CARS siRNA, L-010335-01-0005) and prevalidated control siRNA (Dharmacon, D-001810-10-20) using DharmaFect-4 (Dharmacon, T-2004-03). Note that the siRNA concentration was reduced to 12,5nM for the tRNA ligases to reduce cell toxicity.

ShRNA, lentiviruses and cell transduction
pLKO.1-TSC2 was a gift from Do-Hyung Kim (Addgene plasmid #15478), pLKO-TRC2 (scramble ShRNA, Sigma mission, SH216). For the generation of lentiviruses, 293 cells were cotransfected with with the packaging plasmids (pMDLg/pRRE, pRSV-Rev, and PMD2.G) and the different PLKO plasmids by lipofectamine 2000 for 24 hr. After 24 hr, the medium was replaced with fresh medium. After 48 hr, virus-containing supernatant was collected and centrifuged for 5 minutes at 300g and filtered through 0.45 µm. For the transduction, 2.5 ml were used to transduce LS174T and MCF7 senescent cells in the presence of 4 µg/ml Polybrene (Santa Cruz). After 24h, the media was replaced.

Patient-derived organoids
After informed consent, breast tumors from patients who underwent surgical tumor resection at the ICO Paul Papin Cancer Center were processed through a combination of mechanical disruption and enzymatic digestion by collagenase to generate patient-derived organoids (PDO) as described (32). Briefly, isolated cells were plated in adherent basement membrane extract drops (BME type 2, R&D systems, 3533-010-02) and overlaid with optimized breast cancer organoid culture medium. Medium was changed every 4 days and organoids were passaged every 1-3 weeks. Organoids were treated by 2 cycles of doxorubicin (25 or 50 ng/ml as indicated) for 96 hrs. At the end of the first cycle, the medium was changed and organoids were incubated 3 days without chemotherapy before the second cycle. Following the 2 cycles, the medium was replaced and changed every 3-4 days to analyze chemotherapy escape.

Acknowledgements :
This work was supported by grants from the Ligue Contre le Cancer (Comité du Maine et Loire, du Finistère, de la Loire Atlantique) and the Rotary Club (Maine et Loire).

Data availability: All data are contained within the manuscript

Conflict of Interest : None

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FIGURE LEGENDS

Figure 1: mTOR is necessary for senescence escape

A. Senescence was induced by treating LS174T cells with sn38 as indicated. Cells were then washed with PBS and transfected with a control siRNA or a siRNA directed against p21 for 24 hr and senescence escape was generated by adding 10% FBS.
B. LS174T cells were treated as above and cell extracts were recovered 2 days after p21 depletion. The expression of the indicated proteins was analyzed by western blot (n=3).
C. Number of LS174T emerging clones analyzed after p21 inactivation (n=4, Kolmogorov-Smirnov test * = $p<0.05$).
D. Senescent cells were transfected with a control siRNA or a siRNA directed against p21. Cell extracts were analyzed by SWATH quantitative proteomics and GSEA analysis 2 days after p21 depletion (n=3). Note that these table and experiments are the same as Figure 2A.
E. Validation of mTORCI activation by western blot 2 days after p21 inactivation in LS174T and MCF7 cells (n=3).
F and G. Senescent LS174T cells were transfected with a control siRNA or a siRNA directed against p21 for 24 hr. Cells were then stimulated with 10 % FBS in the presence or absence of mTOR inhibitors (Rapamycin: 5nM, Torin-1: 15nM). The number of emerging clones was evaluated after 10 days (F, n=4 Kolmogorov-Smirnov test * = $p<0.05$). Cell extracts were recovered after 2 days and the expression of the indicated proteins was analyzed by western blot (G, n=3).
H. Senescent cells were generated as above and emergence was induced by a adding 10% FBS. mTORCI activation was analyzed by western blot after 3 days (noted E3 on the figure, n=2 LS174T, n=3 MCF7).
I. Following senescence induction, cells were stimulated with 10% FBS in the presence or absence of mTOR inhibitors. The number of emerging clones was evaluated 10 days later (n=5, Kolmogorov-Smirnov test ** = $p<0.01$).

Figure 2: Inducing an ER stress prevents senescence escape

A. Senescent cells were transfected with a control siRNA or a siRNA directed against p21. Cell extracts were analyzed by SWATH quantitative proteomics and GSEA analysis 2 days after p21 depletion (n=3). Note that these table and experiments are the same as Figure 1D.
B. In the experimental conditions described above, the expression of UPR markers was analyzed by RT-QPCR in LS174T cells (n=4, Kolmogorov-Smirnov test * = $p<0.05$).
C. Senescent MCF7 and LS174T cells were treated with increasing concentrations of tunicamycin as indicated and the number of emerging clones was evaluated 10 days later (n=4, Kolmogorov-Smirnov test, * = $p<0.05$). UPR induction was validated by Western blot 24 hr after the treatment of senescent cells with the lowest concentration of tunicamycin (LS174T: 0.1 µg/ml, n=2; MCF7 :1 µg/ml, n=2).
D. Senescent cells were transfected with a control siRNA or a siRNA directed against CHOP for 24 hr. 10% FBS was added to induce senescence escape and 9 days later the number of emergent clones was analyzed (n=5 for LS174T, 4 for MCF7, Kolmogorov-Smirnov test, * = $p<0.05$, ** = $p<0.01$, note that this experiment has been performed at the same time as Figure 3B).
E. Analysis of the expression of the UPR markers in LS174T and MCF7 senescent cells after 3 days of emergence (noted E3 on the figure, n=3).

Figure 3: mTOR allows senescence escape in the presence of an ER stress

A. After senescence induction, cells were stimulated with 10% FBS in the presence or absence of mTOR inhibitors. Cell extracts were recovered 7 days later and the expression of Bip was analyzed by western blot (n=3).
B. MCF7 and LS174T senescent cells were transfected with a control siRNA or a siRNA directed against CHOP for 24 hr. They were then washed and stimulated with 10% FBS in the presence or absence of mTOR inhibitors. After 9 days, the number of emergent clones was evaluated (n=4, Kolmogorov-Smirnov test, * = $p<0.05$, note that this experiment has been performed at the same time as Figure 2D).
C, D. Following senescence induction, MCF7 and LS174T cells were transduced with a control shRNA or a shRNA directed against TSC2. Cells were then washed and treated or not with tunicamycin (LS174T: 0.1 µg/ml, MCF7: 1 µg/ml, n=4, Kolmogorov-Smirnov test, * = $p<0.05$). TSC2 inhibition was validated by

western blot 2 days after transduction (LS174T n=2, MCF7 n=1) and emergence was then evaluated as above (n=4 to 8, Kolmogorov-Smirnov test, * = $p<0.05$ *** = $p<0.001$).

Figure 4: Down-regulation of tRNA synthesis during senescence

A. Senescent LS174T cells were treated with mTOR inhibitors, and the relative expression of the indicated RNA was analyzed by RT-QPCR after 7 days (n=3).
B. Analysis of the expression of the indicated RNAs 2 days after TSC2 depletion in MCF7 senescent cells by RT-QPCR (n=3).
C. Expression of the indicated RNAs in growing or senescent cells (LS174T n=4, MCF7 n=4, Kolmogorov-Smirnov test, * = $p<0.05$).
D. ChIP analysis of the binding of the type III RNA polymerase in senescent or growing LS174T cells (n=3).
E. Quantitative proteomic analysis of proteins involved in tRNA biogenesis between growing and LS174T senescent cells (n=3).

Figure 5: The LARS and YARS aminoacyl-tRNA synthetases allow senescence escape

A. Analysis of the expression of the indicated RNAs in senescent or emergent cells (after 7 days, LS174T n=5, MCF7, n=4, Kolmogorov-Smirnov test, * = $p<0.05$, **= $p<0.01$).
B. Senescent LS174T cells were transfected with a control siRNA or a siRNA directed against Maf1 or Brf1 during 24 hr and then washed with PBS and stimulated with fresh media. Cell extracts were recovered 2 days after Maf1 or Brf1 depletion and the expression of the indicated RNAs was analyzed by RT-QPCR (n=3).
C. Cells were treated and transfected as described above. Nine days after Maf1 or Brf1 inactivation, the number of emerging clones was analyzed (n=4, Kolmogorov-Smirnov test, * = $p<0.05$).
D. Senescent LS174T and MCF7 cells were transfected with a control siRNA or a siRNA directed against LARS, YARS or CARS for 24 hr. The number of emergent clones was evaluated 9 days later (LS174T n=6, MCF7 n=5, Kolmogorov-Smirnov test, **= $p<0.01$).
E. Analysis of Bip expression by western blot 7 days after BRF1 depletion in LS174T and MCF7 senescent cells (n=3).

Figure 6: Up-regulation of the $tRNA^{Leu}$-CAA and $tRNA^{Tyr}$-GTA in breast organoids that escape chemotherapy

A. Representative image of breast cancer organoids.
B. Analysis by RT-QPCR of proliferative and senescence markers of two breast tumor organoids treated or not with 2 cycles of 96 hr of Doxorubicin (All experiments were performed on two organoids, #1: Doxorubicin 25 ng/ml, #2: Doxorubicin 50 ng/ml).
C. Expression of the indicated RNAs by RT-QPCR of the PDOs at the end of doxorubicin treatment or 10 days later.
D. Evaluation of the viability of MCF7 growing cells treated or not with ABT-263 (5µM) by MTT assay (Left histogram, n=3). Analysis of the number of emerging clones when MCF7 cells were treated with Doxorubicin and ABT-263 for 96 hr (Right histogram, n=4, Kolmogorov-Smirnov test, * = $p<0.05$).
E. Analysis of the viability (TiterGlo) of PDOs treated with ABT-263 (5µM), either in growing conditions or when added in combination with doxorubicin.
F. Analysis of the expression of the indicated RNAs on PDOs at the end of the doxorubicin treatment (E0) and 10 days later (E10).

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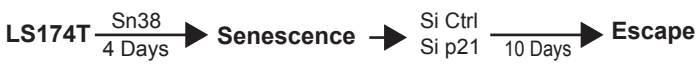

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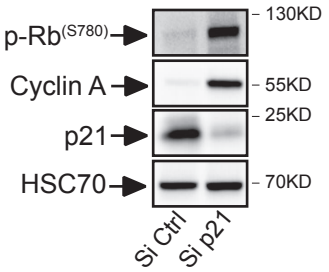

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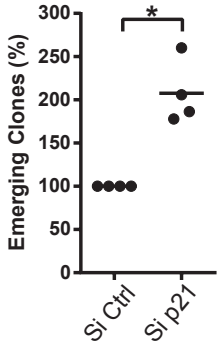

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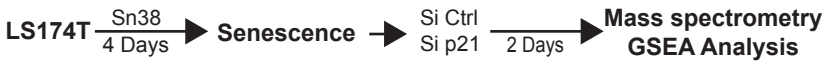
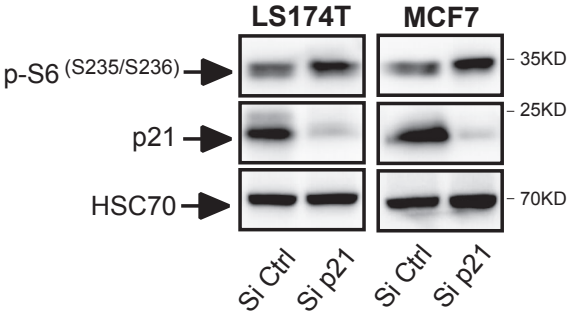


Deregulated Pathways Follwing p21 Inactivation	p-value
HALLMARK_MYC_TARGETS_V1	<0.0001
HALLMARK_E2F_TARGETS	<0.0001
HALLMARK_G2M_CHECKPOINT	<0.0001
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	<0.0001
HALLMARK_MTORC1_SIGNALING	**<0.0001**
HALLMARK_MYC_TARGETS_V2	0.0038461538
HALLMARK_DNA_REPAIR	0.020491803
HALLMARK_MITOTIC_SPINDLE	0.35458168
HALLMARK_UV_RESPONSE_UP	0.35559922
HALLMARK_GLYCOLYSIS	0.6627451

E.



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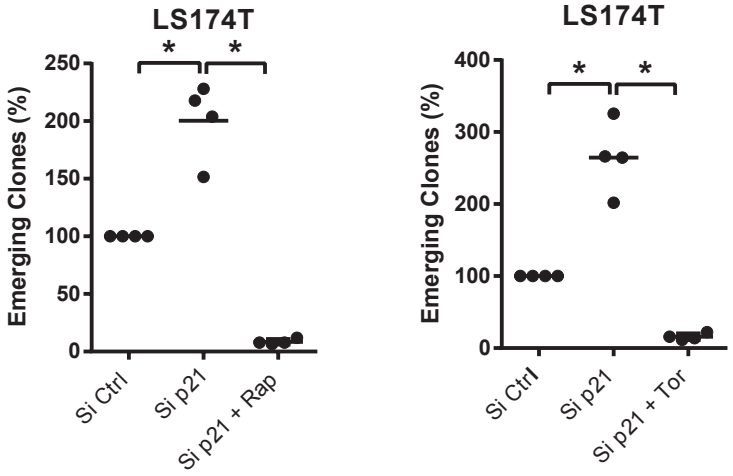

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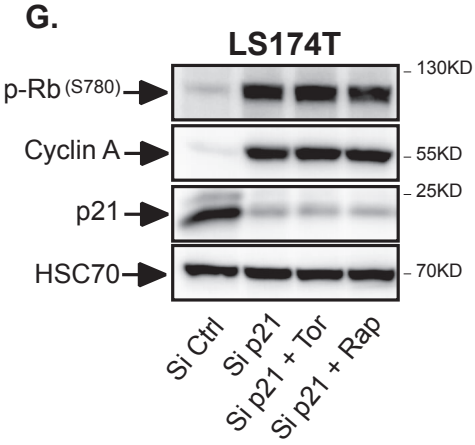

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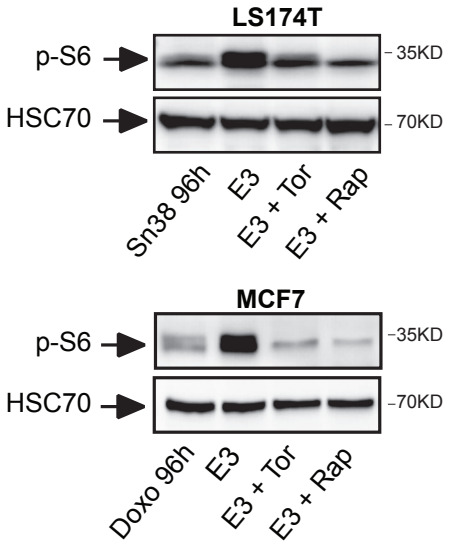

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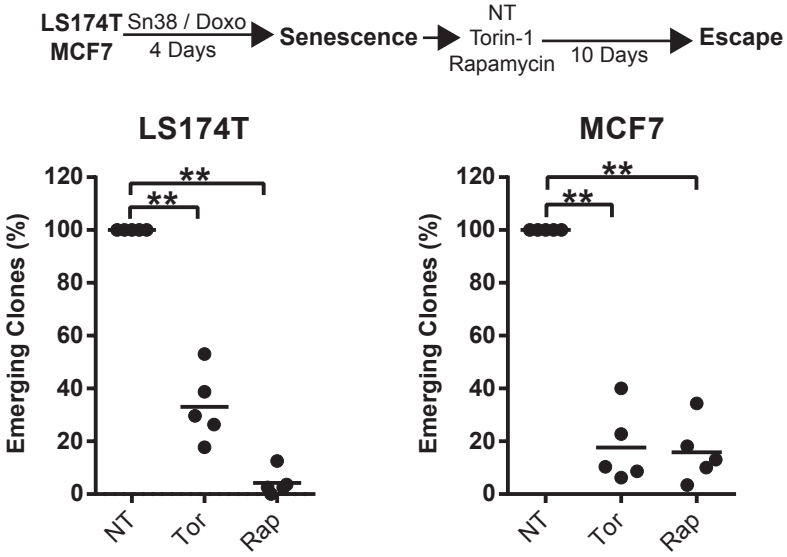
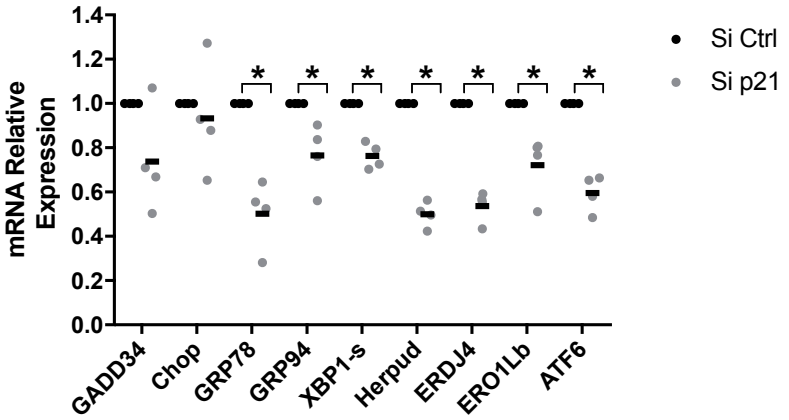


Figure 1

A.

Deregulated Pathways Follwing p21 Inactivation	p-value
HALLMARK_MYC_TARGETS_V1	<0.0001
HALLMARK_E2F_TARGETS	<0.0001
HALLMARK_G2M_CHECKPOINT	<0.0001
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	**<0.0001**
HALLMARK_MTORC1_SIGNALING	<0.0001
HALLMARK_MYC_TARGETS_V2	0.0038461538
HALLMARK_DNA_REPAIR	0.020491803
HALLMARK_MITOTIC_SPINDLE	0.35458168
HALLMARK_UV_RESPONSE_UP	0.35559922
HALLMARK_GLYCOLYSIS	0.6627451

B.



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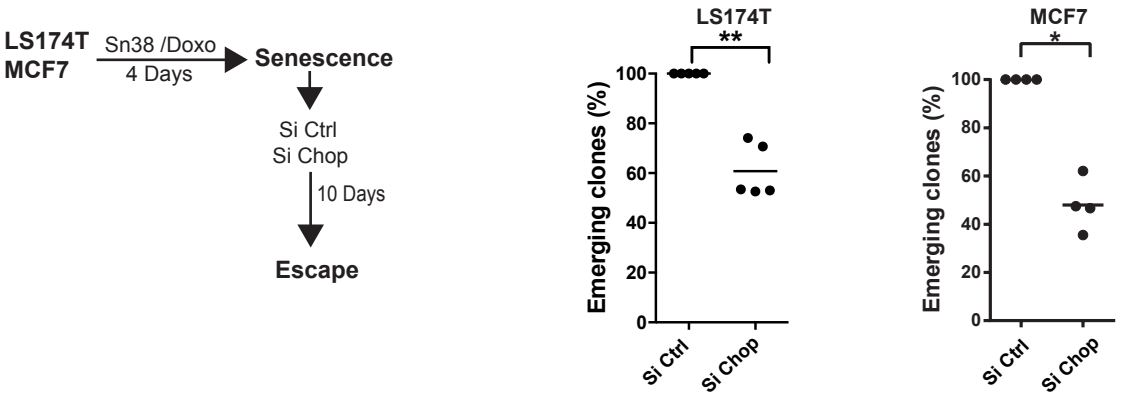

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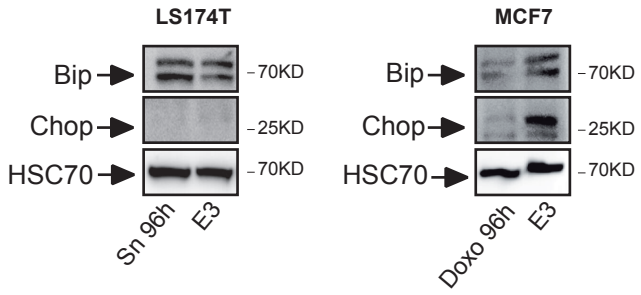
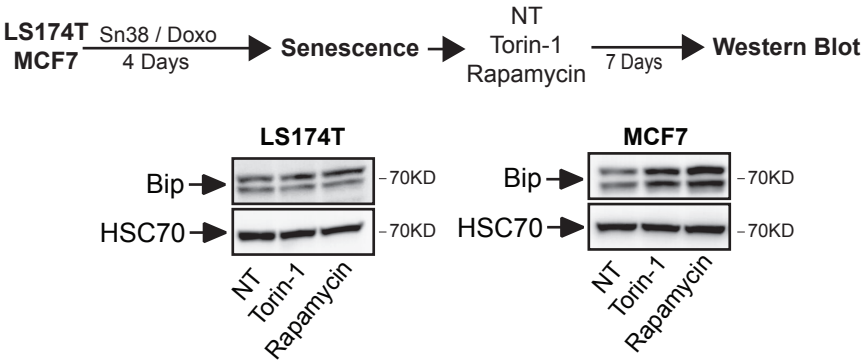


Figure 2

A.



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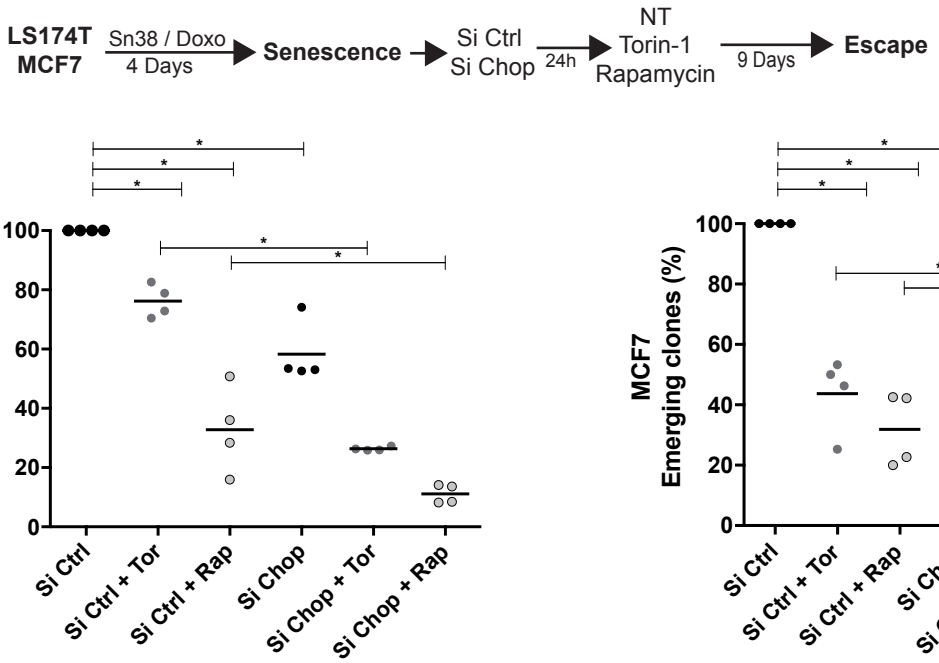

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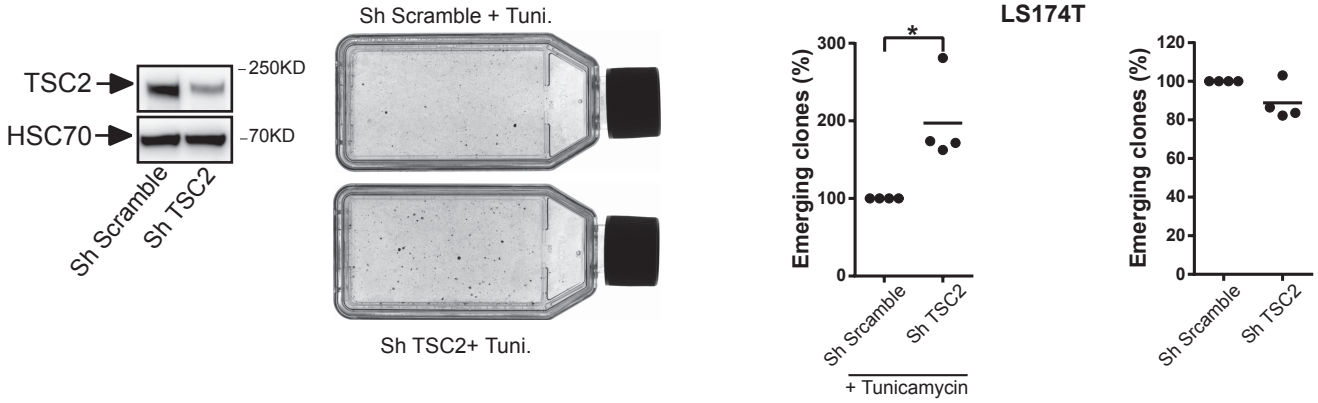

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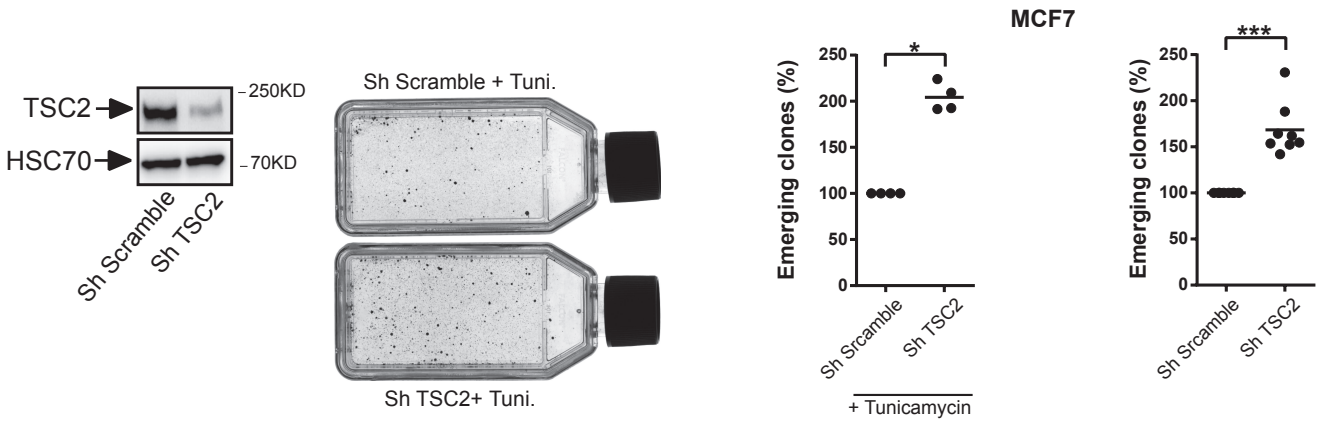
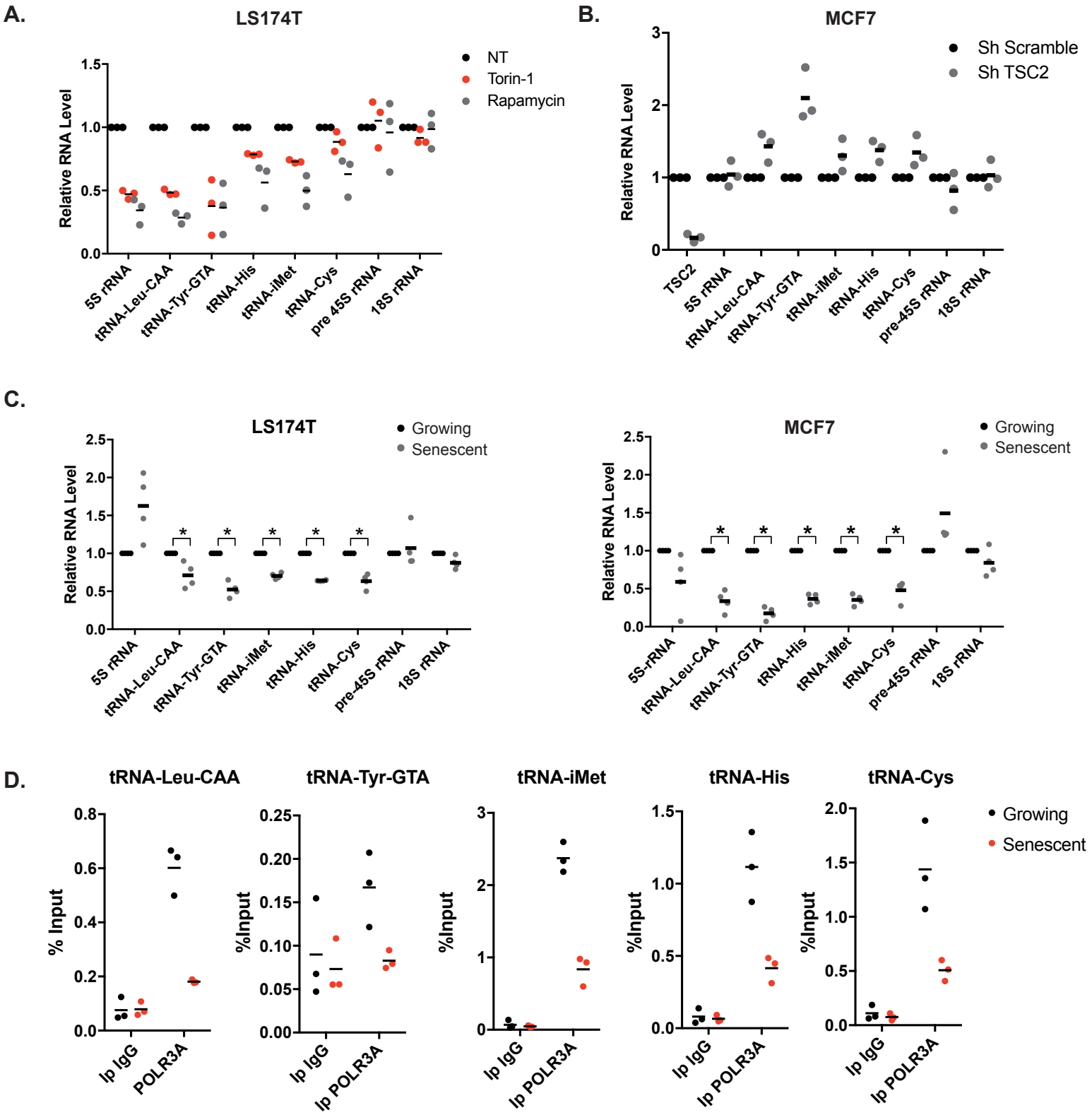


Figure 3



E.

LS174T —NT / Sn38, 4 Days→ Mass Spectrometry

Name	Gene	p-value	Fold Change Sn96h /NT
Alanine--tRNA ligase, cytoplasmic	AARS	6,68E-07	0,364316414
Tyrosine--tRNA ligase, cytoplasmic	YARS	6,82E-08	0,445137351
Serine--tRNA ligase, cytoplasmic	SARS	3,65E-10	0,454269252
Glycine--tRNA ligase OS	GARS	2,25E-05	0,530127785
tRNA methyltransferase 112 homolog	TRMT112	0,00013	0,553876549
Leucine--tRNA ligase, cytoplasmic	LARS	0,00035	0,585097856
Tryptophan--tRNA ligase, cytoplasmic	WARS	0,00412	0,646939838
Asparagine--tRNA ligase, cytoplasmic	NARS	1,18E-07	0,760747593
Bifunctional glutamate/proline--tRNA ligase	EPRS	0,00016	0,776409787
Phenylalanine--tRNA ligase alpha subunit	FARSA	0,00011	0,795828776
Isoform 3 of Cysteine--tRNA ligase, cytoplasmic	CARS	0,02794	0,800776054
Aspartate--tRNA ligase, cytoplasmic	DARS	0,00879	0,803614741
Isoleucine--tRNA ligase, cytoplasmic	IARS	2,06E-06	0,808479902
Lysine--tRNA ligase	KARS	0,00183	0,833207177
Valine--tRNA ligase	VARS	0,00211	0,847178975
Phenylalanine--tRNA ligase beta subunit	FARSB	0,04808	0,907427017
Arginine--tRNA ligase, cytoplasmic	RARS	0,00874	1,135050804
CCA tRNA nucleotidyltransferase 1, mitochondrial	TRNT1	0,03978	1,278298875
Methionine--tRNA ligase, mitochondrial	MARS2	0,03271	1,299050634
Aspartate--tRNA ligase, mitochondrial	DARS2	1,20E-06	1,328844826
Histidine--tRNA ligase, cytoplasmic	HARS	0,01393	1,560598091

Figure 4

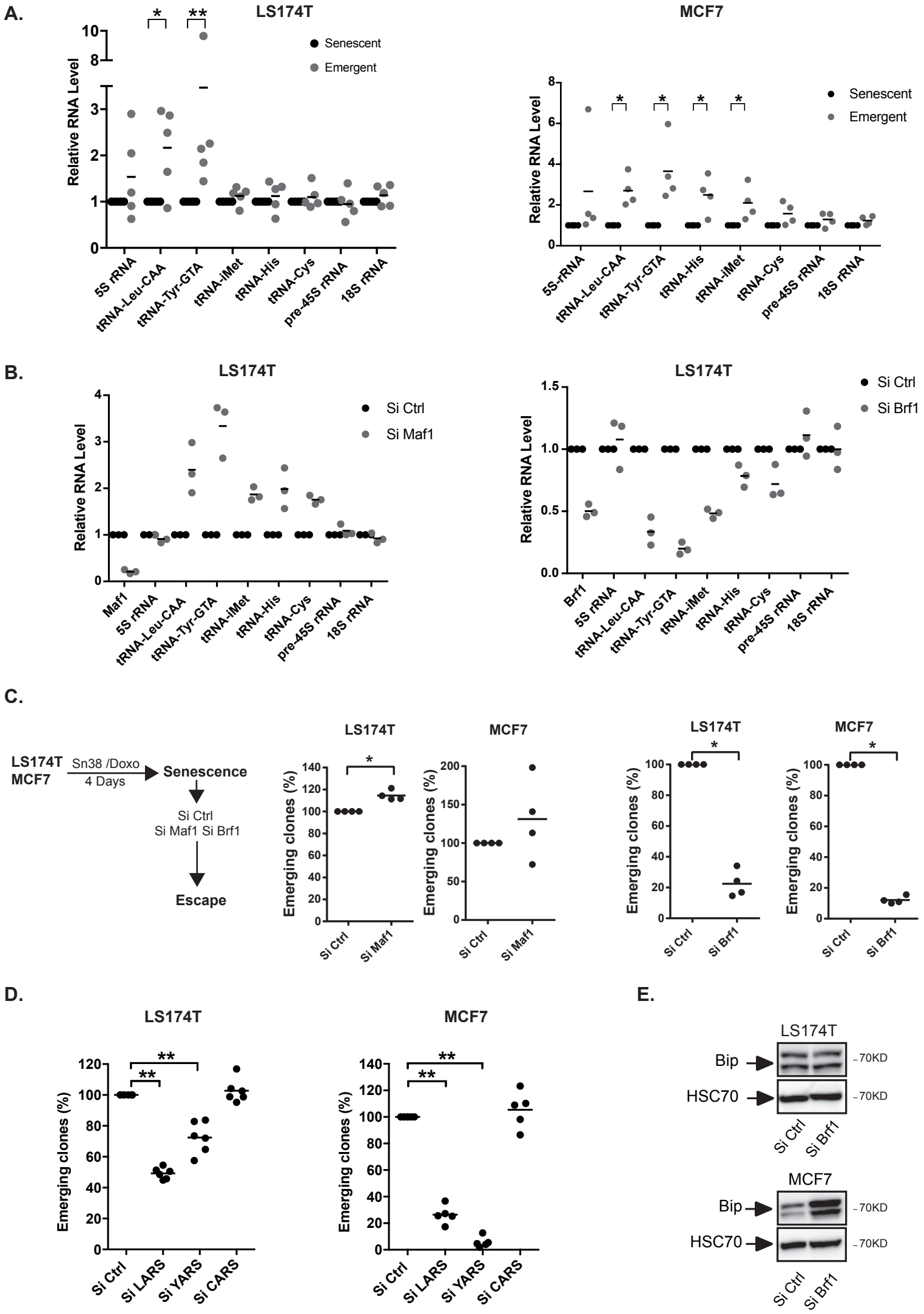
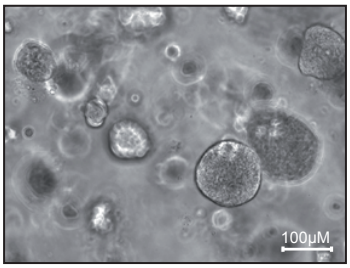
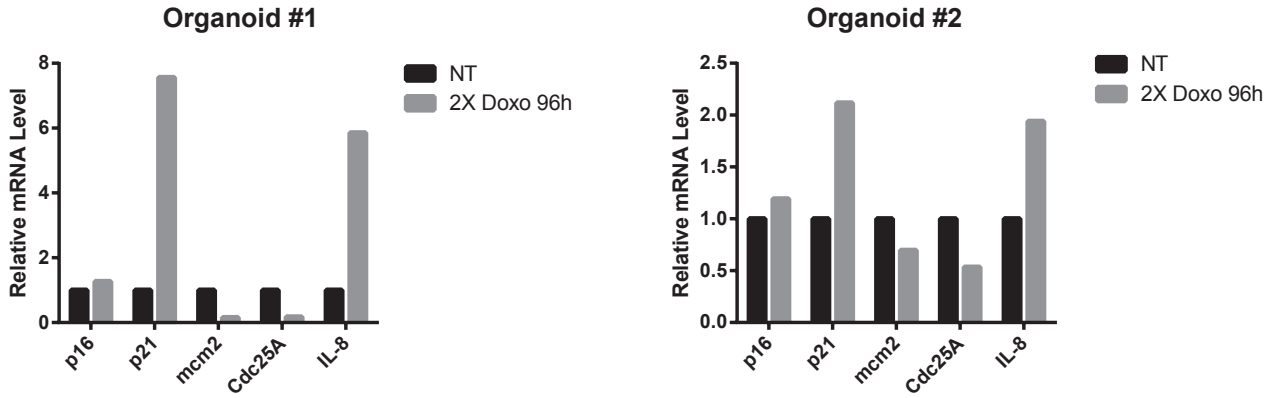

A.
LS174T
MCF7
Senescent
Emergent
Relative RNA Level
5S rRNA
tRNA-Leu-CAA
tRNA-Tyr-GTA
tRNA-iMet
tRNA-His
tRNA-Cys
pre-45S rRNA
18S rRNA
B.
Si Ctrl
Si Maf1
Si Brf1
Maf1
Brf1
C.
LS174T
MCF7
Sn38 /Doxo
4 Days
Senescence
Si Ctrl
Si Maf1 Si Brf1
Escape
Emerging clones (%)
D.
Si LARS
Si YARS
Si CARS
E.
Bip
HSC70
70KD

Figure 5

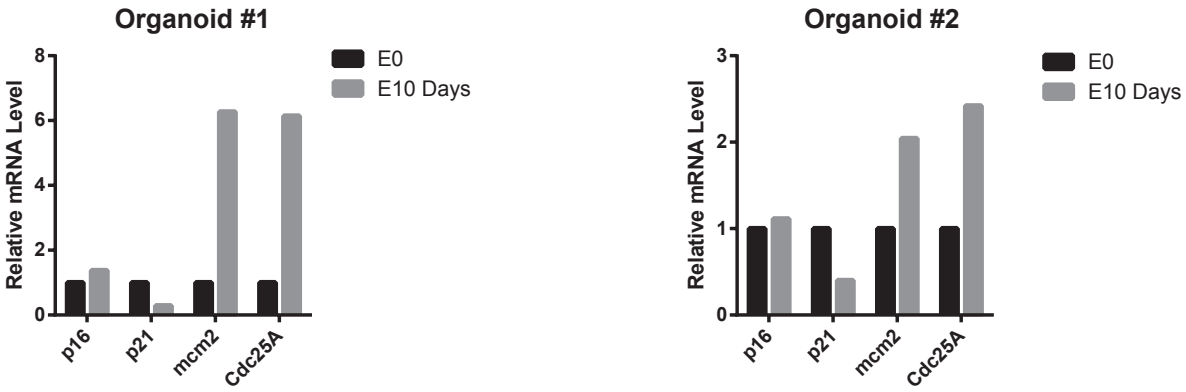
A.



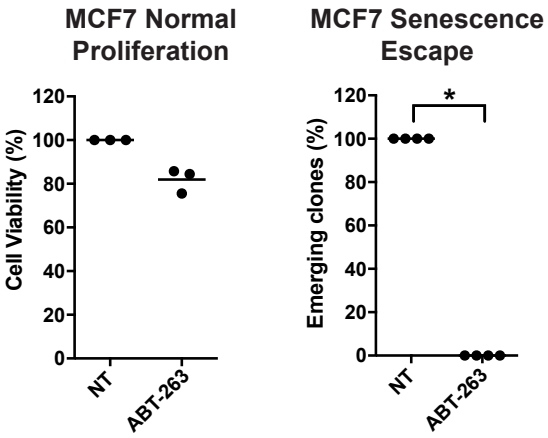
B.



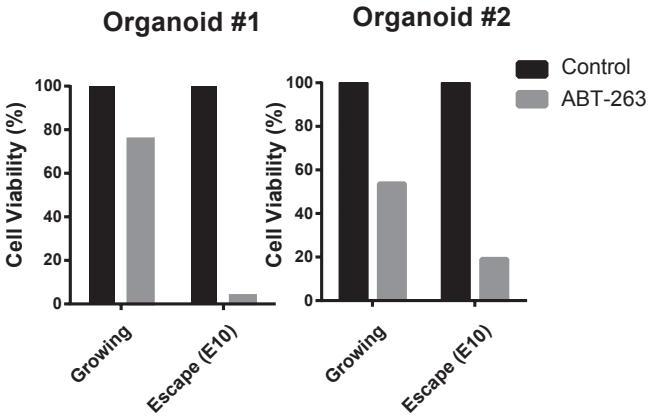
C.



D.



E.



F.

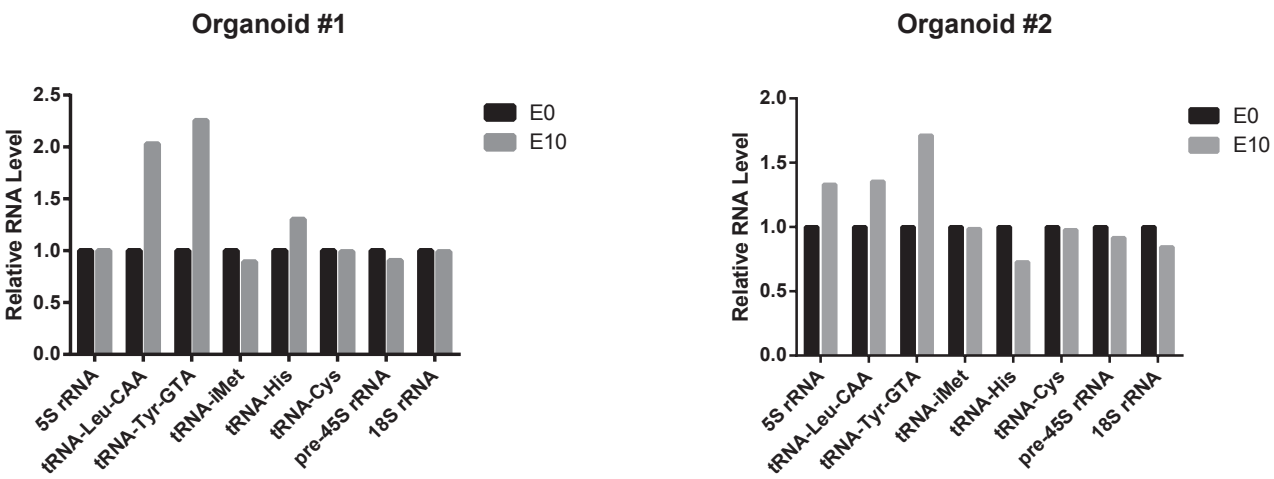
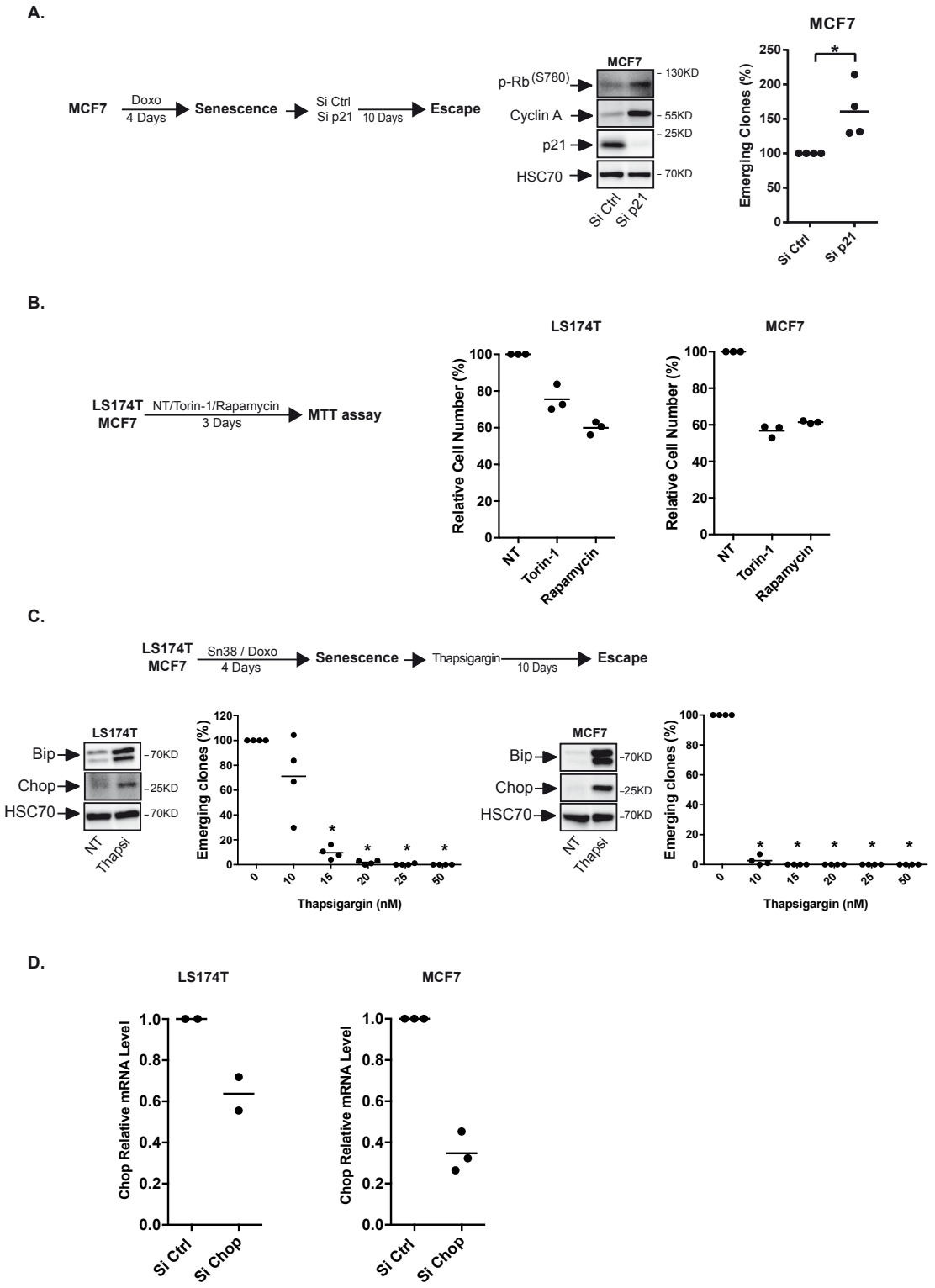
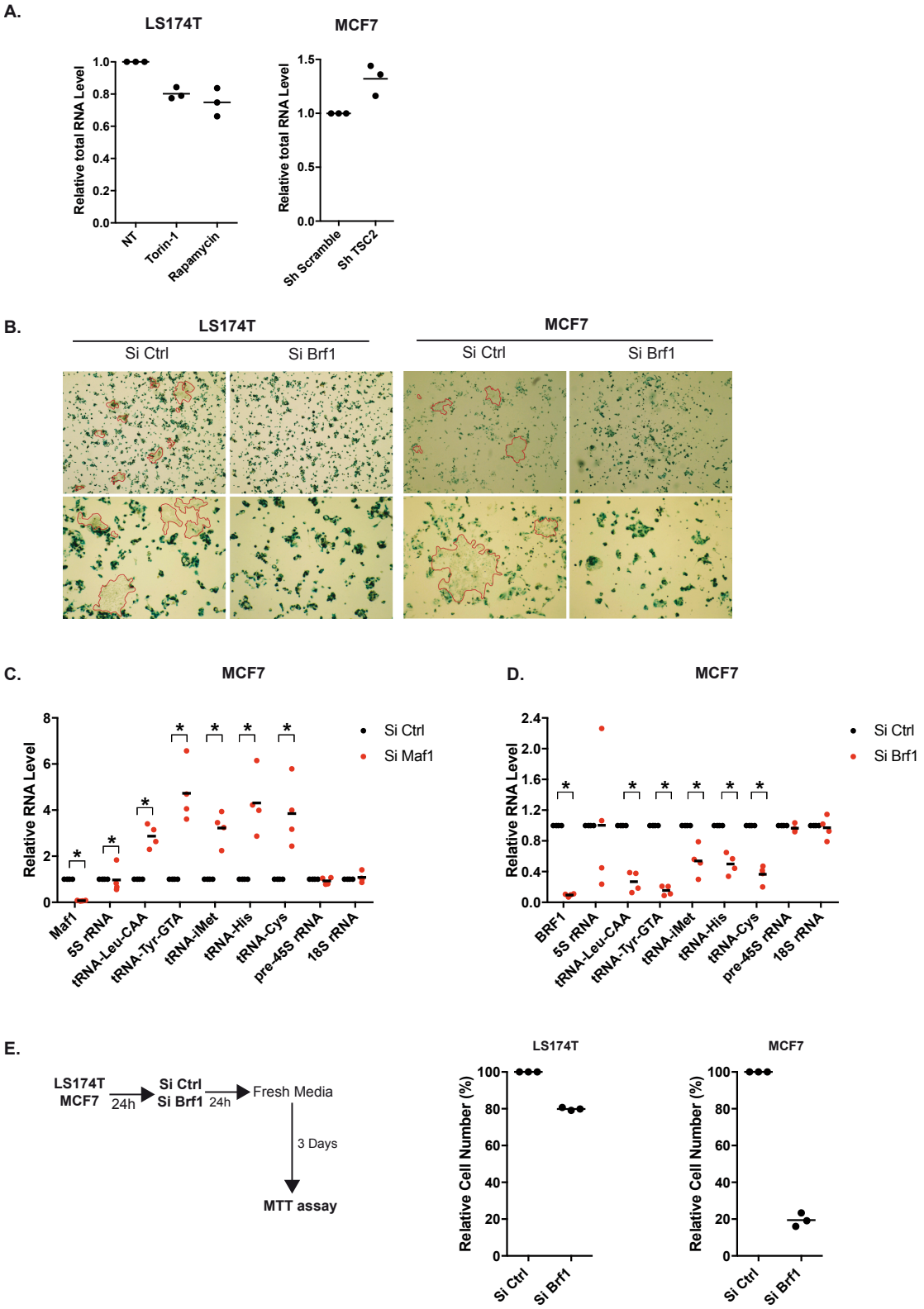


Figure 6



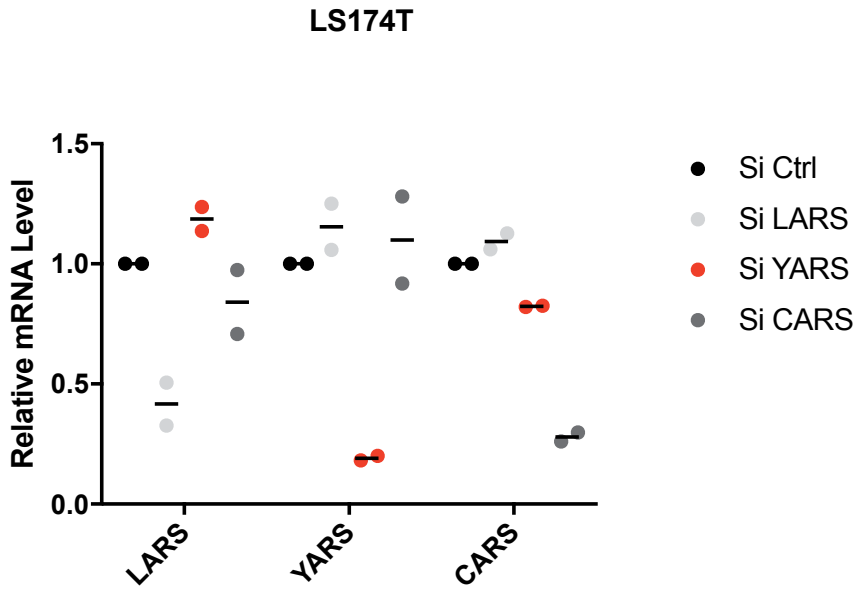
Supplementary Figure 1

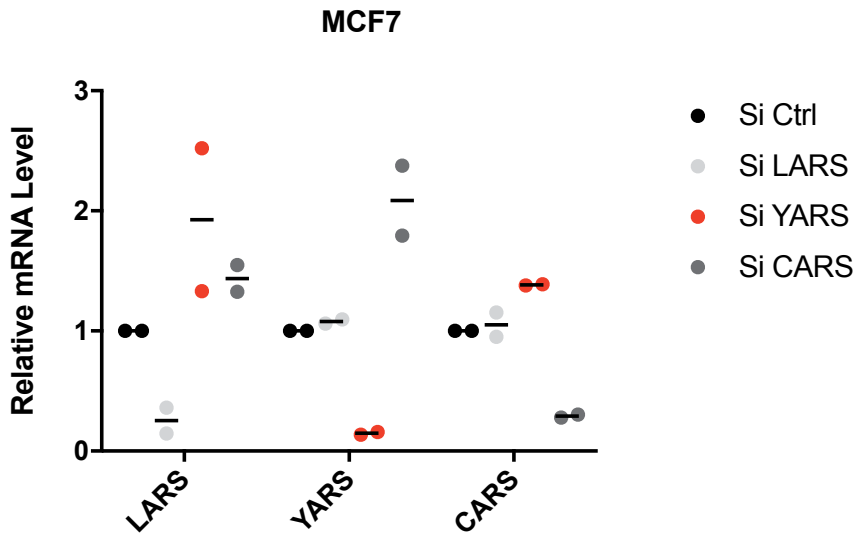
A. Senescent MCF7 cells were transfected with a control siRNA or a siRNA directed against p21 for 24 h and persistant cells were then generated by adding 10% FBS. p21 down-regulation as well as the expression of cyclin A and Rb phosphorylation were evaluated by Western blot two days after the depletion. Emergence was evaluated after 10 days (n=3 for the western, 4 for emergence, Kolmogorov-Smirnov test, * = $p<0.05$) **B**. LS174T and MCF7 cells were treated or not with mTOR inhibitors (Torin-1: 15nM; Rapamycin: 5nM), and cell viability was analyzed by MTT assay after 3 days. (n=3). **C**. Senescent cells were treated with increasing concentrations of thapsigargin and the number of emerging clones was evaluated 10 days later (n=4, Kolmogorov-Smirnov test,* = $p<0.05$). The induction of UPR was validated by Western blot after 24h using a 10 nM concentration (n=2). **D**. After senescence induction, LS174T and MCF7 cells were transfected with a control siRNA or a siRNA directed against Chop for 24 h. The efficiency of the depletion was confirmed two days later by RT-QPCR (n=2 for LS174T, n=3 for MCF7).



Supplementary Figure 2

A. Quantification of total RNA level in senescent LS174T cells two days after mTOR inhibition (left, n=3) or in senescent MCF7 cells two days after TSC2 inhibition (right, n=3). **B.** Representative images of SA-β galactosidase staining 7 days after BRF1 inactivation in LS174T and MCF7 senescent cells (n=3). Growing persistant cells are underlined in red. **C and D**. After senescence induction, MCF7 cells were transfected with a control siRNA or a siRNA directed against Maf1 (**C**) or Brf1 (**D**) during 24 h, cell extracts were recovered after 2 days of emergence and RNA expression was analyzed by RT-QPCR (n=4, Kolmogorov-Smirnov test, * = p<0.05). **E**. LS174T and MCF7 were transfected with a control siRNA or a siRNA directed against BRF1 during 24 h. Cells were then seeded in 96h plates in fresh media and proliferation was evaluated after 3 days using MTT assays (n=3+-/sd).





Supplementary Figure 3

Senescent LS174T or MCF7 cells were transfected with a control siRNA or a siRNA directed against the indicated aminoacyl-tRNA synthetases (ARS) involved in the tRNA ligation of the leucine (LARS), tyrosine (YARS) and cystein (CARS) amino acids. 10% FBS was then added for two days and mRNA expression was analyzed by RT-QPCR (n=2).