Alteration of ribosome function upon 5-fluorouracil treatment favors cancer cell drug-tolerance

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Mechanisms of drug-tolerance remain poorly understood and have been linked to genomic but also to non-genomic processes. 5-Fluorouracil (5-FU), the most widely used chemotherapy in oncology is associated with resistance. While prescribed as an inhibitor of DNA replication, 5-FU alters all RNA pathways. Here, we show that 5-FU treatment leads to the production of fluorinated ribosomes exhibiting altered translational activities. 5-FU is incorporated into ribosomal RNAs of mature ribosomes in cancer cell lines, colorectal xenografts, and human tumors. Fluorinated ribosomes appear to be functional, yet, they display a selective translational activity towards mRNAs depending on the nature of their 5′-untranslated region. As a result, we find that sustained translation of IGF-1R mRNA, which encodes one of the most potent cell survival effectors, promotes the survival of 5-FU-treated colorectal cancer cells. Altogether, our results demonstrate that “man-made” fluorinated ribosomes favor the drug-tolerant cellular phenotype by promoting translation of survival genes.
Partial response to chemotherapy leads to disease resurgence. Upon treatment, a subpopulation of cancer cells, called drug-tolerant persistent cells, displays a transitory drug tolerance that leads to treatment resistance1,2. Though drug-tolerance mechanisms remain poorly understood, they have been linked to non-genomic processes, including epigenetics, stemness and dormancy2–4.

Translation regulation plays a major role in controlling gene expression and contributes to disease emergence including cancer5–7. Within ribosomes, ribosomal RNAs (rRNAs) play a central role in the translation process, by monitoring codon-anticodon recognition, coordinating ribosomal subunit activity and catalyzing peptide bond formation through their ribozyme activity. rRNAs contain over 200 naturally occurring chemical modifications, which stabilize rRNA structures and create additional molecular interactions not provided by non-modified nucleotides7–9. Chemical modifications of rRNAs were shown to directly contribute to translational regulation8,10,11. We, and others, showed that rRNA chemical modifications contribute to the fine-tuning of ribosome functions and to the modulation of ribosome translational activity of ribosomes in cancer cells12–15.

5-fluorouracil (5-FU) is the most widely used chemotherapy in cancer treatment. 5-FU efficacy is partial and often associated with resistance16. While discovered and used as an inhibitor of DNA replication, 5-FU alters all RNA pathways16–20. Indeed, 5-FU treatment results in 5-fluorouridine (5-Urd) incorporation into various types of cellular RNAs including the precursor of rRNA16. However, the consequences of 5-FUrd incorporation into ribosomal RNA precursor on ribosome production and functioning have so far not been analyzed, neither has its impact on cellular phenotype. Here, we show that 5-FU is incorporated into rRNAs of mature ribosomes in several models including cancer cell lines, colorectal mouse xenografts, and human colorectal tumor samples. 5-FU-containing ribosomes appear to be functional, yet, they display a selective translational activity towards mRNA subsets depending on the nature of their 5′-untranslated region. We find that upon 5-FU treatment, translation of the mRNA of the pro-survival IGF-1R gene is sustained, and promotes the survival of 5-FU-treated colorectal cancer cells. Altogether, our results demonstrate that fluorinated ribosomes favor the drug-tolerant cellular phenotype by promoting the translation of survival genes.

Results

Ribosome production is partially maintained upon 5-FU treatment. Previous work indicated that at a high concentration, 5-FU alters ribosome biogenesis without inhibiting pre-rRNA synthesis20,21. To further investigate this, we treated colorectal cancer HCT116 cells with clinically relevant concentrations of 5-FU (10–50 µM)22,23, which resulted in growth inhibition and cell death (24 and Supplementary Fig. 1a). Within this concentration range, 5-FU treatment resulted in enlarged nucleoli, absence of nucleolar cap formation and absence of dispersion of nucleolar markers, as opposed to cells treated with the RNA Pol I inhibitor actinomycin D (Fig. 1a and Supplementary Fig. 1b, c). Such nucleolar restructuring reveals an alteration of ribosome biogenesis albeit without pre-rRNA synthesis inhibition, and was confirmed by transmission electron microscopy (TEM) (Supplementary Fig. 1d). Consistently, 47 S/45 S pre-rRNA levels, analyzed by Northern blotting and RNA fluorescent in situ hybridization (FISH), were unchanged following 5-FU treatment confirming that 5-FU did not affect RNA Pol I activity (Fig. 1b and Supplementary Figs. 1e, 2a, b).

Northern blot analysis also confirmed that ribosome maturation at post-transcriptional steps was altered, and revealed that the pre-rRNA processing was impaired at the cleavage stage at site 2 (Supplementary Fig. 2a–c). Yet, despite this effect, the late pre-rRNA intermediates leading to 18 S and 28 S rRNA were still detected (Supplementary Fig. 2c) suggesting that ribosome production was in part maintained. This was confirmed by [35S]pulse-chase experiments that showed that ribosomes are produced at significant levels for up to 48 h under 5-FU treatment (Fig. 1c and Supplementary Fig. 2d). Thus, at a clinically relevant concentration of 5-FU, each step of ribosome processing is able to proceed, despite the stringent quality control, thus allowing ribosome production to be maintained at a substantial level.

5-FU incorporates into ribosomes. 5-FU was previously shown to be converted to 5-FUTP and incorporated into RNA16. We therefore wondered whether ribosomes produced and exported to the cytoplasm in treated cells contained 5-FUrd within their rRNAs. To test this, we developed a quantitative liquid chromatography—mass spectrometry—high-resolution mass spectrometry (LC-MS-HRMS) approach that allowed us to determine the number of 5-FUrd incorporated into rRNA of cytoplasmic ribosomes purified at high stringency on a 500 mM KCl sucrose cushion (Fig. 2a, see methods for details25). We found that HCT116 ribosomes contained significant amounts of 5-FUrd, ranging from 7 to 14 5-FUrd molecules per ribosome following 24 h of treatment with 5–100 µM of 5-FU (Fig. 2b). We performed two experiments to rule out that the 5-FUrd signal came from nonribosomal RNA. First, we measured 5-FUrd from gel-purified 18 S and 28 S rRNA (Supplementary Fig. 3a) to isolate 18 S and 28 S rRNA from the other cellular RNAs by cutting out the corresponding bands from the gel. Second, because direct purification of ribosomes on a 500 mM KCl sucrose cushion might result in minor mRNA contamination, we measured 5-FUrd from ribosomal subunits dissociated with puromycin (Supplementary Fig. 3b, c). Our data show that puromycin treatment of the ribosomal subunit fully dissociated a small number of subunits that were still assembled after direct purification on the 500 mM KCl sucrose cushion (Supplementary Fig. 3b). Yet the number of 5-FUrd per ribosome was identical in ribosomal subunits separated with puromycin compared to ribosomal subunits directly purified on the 500 mM KCl sucrose cushion (Supplementary Fig. 3c), indicating that the data obtained by our method reflects 5-FUrd incorporation into rRNA. Next, we extended the analysis to additional cancer cell lines: (i) CRC cells characterized by different molecular profiles reflective of the pathology displaying the different combination of KRAS, BRAF and TP53 mutations, as well as the microsatellite instability (MSI) and CIN statuses (Supplementary Table 1) and (ii) cell lines from triple-negative breast cancer and (ii) pancreatic cancer. 5-FUrd was incorporated into rRNA of cytoplasmic ribosomes purified from all tested cell lines after 24 h of treatment with 10 µM 5-FU (Fig. 2c). Altogether, these data demonstrate that upon 5-FU treatment, ribosomes containing fluorinated rRNA are fully assembled and exported to the cytoplasm, revealing that presence of 5-FUrd is tolerated by the quality control systems of the cell.

Next, we investigated whether fluorinated ribosomes could be found within tumors in vivo. First, we analyzed rRNA from HCT116 xenografts established in nude mice. 5-FU treatment efficacy was evidenced by a decrease in tumor growth (Supplementary Fig. 3d). 5-FUrd was detected in mature rRNA purified from tumor cells collected after the last treatment at levels close to those observed in cultured cells (Fig. 2d). We extended this observation to xenografts similarly established with HT29 and SW480 cell lines (Fig. 2e, f). Thus, 5-FU incorporation into ribosomes can be replicated in a common

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xenografted animal model. Finally, we analyzed rRNA of colorectal tumor cells from patients treated with 5-FU-based therapies, using large RNA quantities to optimize detection (Supplementary Fig. 3e). Of the 5 samples tested from 5-FU-treated patients, 5-FUrd was detected in rRNA from 2 patients (3.80 and 4.50 5-FUrd per ribosome respectively; Fig. 2g), a patient receiving no 5-FU served as a negative control. Altogether, these data show that 5-FUrd is largely incorporated into rRNA of cells treated with 5-FU, and that 5-FU-based chemotherapy leads to the production of fluorinated ribosomes within tumor cells both in animal models and in humans.
Altered translation by fluorinated ribosomes. Because rRNAs and their post-transcriptional chemical modifications play a central role in ribosome functioning, and because 5-FU induces changes in translational regulation, we postulated that fluorinated ribosomes may display modified translational activity. To investigate this, we first considered whether fluorinated ribosomes could be recruited onto mRNA during translation, by analyzing the rRNA 5-FURd content in actively translating ribosomes isolated by sucrose gradient (Fig. 3a). Next, we evaluated whether the incorporation of 5-FU into rRNA impacts the translational capacity of ribosomes. We used our recently developed in vitro hybrid translation assay, in which only ribosomes have been exposed to 5-FU, in order to evaluate the activity of purified fluorinated ribosomes in a controlled setting (Fig. 3b). To gain insight into the changes in ribosome activity the translational capacity of fluorinated ribosomes was assessed using a set of luciferase reporter mRNAs, representing different functional mRNAs and whose translation relies on different 5′UTR: (i) mRNAs of two housekeeping genes containing short 5′UTR from globin and GAPDH mRNAs, (ii) mRNA of two cancer-promoting genes containing long and structured 5′UTR from IGF-1R and c-Myc mRNAs, and (iii) mRNA of two viral genes containing long and structured uncapped 5′UTR from cricket paralysis virus (CrPV) and encephalomyocarditis virus (EMCV), which initiate translation through an internal ribosome entry site (IRES). The results showed first that fluorinated ribosomes were not impaired for translation. Second that they displayed a selective translation initiation efficacy that differed from that of control ribosomes, and varied according to the nature of the 5′UTR upstream of the reporter mRNA used (Fig. 3c). Indeed, globin and GAPDH were less efficiently translated, a result that is consistent with lower overall protein synthesis in 5-FU treated cells (Fig. 3a) and Supplementary Fig. 5a). Moreover, reporter mRNAs containing IGF-1R and c-Myc 5′UTR were more efficiently translated by fluorinated ribosomes. These differences suggest that translation efficiency varies according to the nature of the 5′UTR, indicating that the initiation step of translation differs for fluorinated ribosomes compared to normal ribosomes. To consolidate this hypothesis, translation was tested on a mRNA carrying the CrPV intergenic IRES, an element that directly binds to the ribosome and initiates translation without any cellular translation initiation factors (eIFs). Fluorinated ribosomes displayed a decrease in translational activity on CrPV mRNA, strongly supporting that fluorinated ribosomes are structurally or functionally different (Fig. 3d). This defect in translation initiation from the CrPV intergenic IRES was not strictly related to cap-independent initiation mechanisms since fluorinated ribosomes were more efficient at translating an EMCV IRES containing mRNA, another cap-independent translation initiation model (Fig. 3d).

Next, we focused on IGF-1R 5′UTR which is one of the longest 5′UTR in the human genome and contains several regulatory elements, the activity of which could be influenced by 5-FU, including an IRES element. First, we mapped the IRES element of IGF-1R 5′UTR by carrying out a series of deletions (Supplementary Fig. 5b, c) and localized it in the last 84 nucleotides (region 954-1040), and then verified that no IRES activity was carried out by the 1-953 region, an observation consistent with the previous studies. When evaluated in 5-FU treated cells, the IRES activity of IGF-1R 5′UTR was increased both when the full-length 5′UTR or only the minimal IRES (region 954-1040) were used (Fig. 3e). In contrast no effect was observed for the 1-953 region (ΔIRES). In addition, c-MYC 5′UTR IRES activity was not impacted (Supplementary Fig. 5d), suggesting that the activity of some IRES elements is not impacted by 5-FU and that regulation of c-MYC translation involves another mechanism. To determine whether the IRES element could play a dominant role in IGF-1R translation regulation, we tested the same construct using a monocistronic assay, where the 5′UTR is directly placed at the 5′ of the reporter mRNA and capped (Fig. 3f). We found that both the full-length 5′UTR and the minimal IRES had significant increased translational activity upon 5-FU treatment, and that the activity of the 1-953 region of IGF-1R 5′UTR and that of GAPDH 5′UTR were not significantly affected by 5-FU, although we observed a tendency towards a decrease at high 5-FU concentrations (Fig. 3f).

Altogether, these experiments demonstrate that 5-FU incorporation into rRNA modifies the ability of ribosomes to initiate mRNA translation from different 5′UTR. Taken together, they highlight that fluorinated ribosomes might contribute to 5-FU-induced translational reprogramming that we previously observed.

IGF-1R promotes 5-FU drug tolerance. The data above suggest that fluorinated ribosomes favor translation of selected mRNAs, including genes such as IGF-1R and c-Myc, that may promote early cell survival and lead to resistance. We focused on IGF-1R, a gene that plays a major role in tumorigenesis and whose contribution to cell survival has been largely demonstrated in various models including colorectal cancer. Because 5-FU treatment induces a decrease in global protein synthesis, we initially evaluated whether IGF-1R mRNA translation was also impacted by 5-FU treatment in HCT116 cells. IGF-1R mRNA translation efficacy was assessed by measuring the recruitment of cytoplasmic mRNA into the heavy polysome fraction of control and 5-FU-treated cells (Fig. 4a and Supplementary Fig. 6). Our data show that the fraction of IGF-1R mRNAs associated with heavy polysomes was maintained in 5-FU treated cells, while that of actin and GAPDH mRNAs decreased, indicating that translation of IGF-1R mRNA is selectively favored compared to housekeeping mRNAs, a result consistent with in vitro translation data obtained with reporter mRNAs (Fig. 3c). At the protein level, IGF-1R increased compared to relatively stable levels of Actin, GAPDH and Histone H3 proteins after 24 h and 48 h of 5-FU treatment (Fig. 4b, c). Next, to determine whether the IGF-1-IGF-1R pathway contributes to the survival of CRC cells exposed to 5-FU, cells were first treated with 5-FU for 24 h or 48 h, and were subsequently treated with...
IGF-1. Cell proliferation was monitored over 5 days, and revealed that, while IGF-1 had no impact on control cells, it improved the growth of cells treated with 5-FU (Fig. 4d, e, and Supplementary Fig. 7a). To further validate our findings, HCT116 cells were co-treated with 5-FU and the IGF-1R kinase inhibitor NVP-AEW541, and cell response was monitored by viability assay (Fig. 4f). Inhibition of IGF-1R induced a stronger decrease in cell survival when cells were co-treated with 5-FU compared to untreated cells demonstrating that 5-FU treated cells relied more on an active IGF-1/IGF-1R pathway than untreated cells, and that the IGF-1/IGF-1R pathway is necessary for optimal cell tolerance to 5-FU. Overall, our results unveil that the IGF-1/IGF-1R
pathway plays a role in the survival of a cell subpopulation upon 5-FU treatment, and strongly support that the 5-FU driven maintenance of IGF-1R synthesis contributes to this mechanism.

5-FU treatment leads to translational upregulation of cell survival-associated mRNAs. Next, we wondered whether the changes in translation induced by 5-FU could promote cellular functions associated with cell survival or drug tolerance. The impact of 5-FU on cellular mRNA translation was evaluated by polysome profiling on cells treated with 10 µM or 50 µM 5-FU for 24 h (Supplementary Fig. 8a, Supplementary Fig. 9). Briefly, mRNAs associated with actively translating ribosomes (i.e., polysomes) were purified (Supplementary Fig. 8b) and analyzed by RNA-Seq to compare the polysomal level of each transcript between 5-FU treated and untreated cells (Fig. 5a, b, Supplementary Data 1). We found that 5-FU treatment altered translation for a fraction of mRNA, representing about 7% and 10% of analyzed mRNAs, in 10 µM and 50 µM 5-FU treated cells respectively (Fig. 5a, b), with a majority of translationally altered mRNA being upregulated (10 µM, 702 up vs. 275 down; 50 µM: 937 up vs. 477 down, corresponding to 72% and 66% respectively). An increased number of differentially translated mRNAs was observed between 10 µM and 50 µM 5-FU conditions (977 vs 1414), most of these mRNAs being common to these two conditions, indicating a dose-dependent response (Fig. 5c). A Gene Ontology (GO) enrichment analysis performed on all translationally altered mRNAs (Fig. 5d, Supplementary Fig. 8c and Supplementary Data 2) revealed similar GO-term enrichment for 10 µM and 50 µM treated cells. As expected, enrichment in p53 pathway, DNA damage response and apoptosis were significantly represented, which reflects the well-known cytotoxic activity of 5-FU. Next, a clustering of GO terms, which groups GO terms related to the same functions (Fig. 5e, f), revealed that in response to 50 µM 5-FU, the translationally downregulated mRNAs mainly correspond to gene classes reflecting cell proliferation, such as cell cycle or cell and organelle biogenesis; an observation also consistent with a stress-induced proliferation arrest (Fig. 5e, Supplementary Data 3). Interestingly, a majority of translationally altered mRNAs were upregulated both in 10 µM and 50 µM 5-FU conditions (Fig. 5a, b). These translationally upregulated mRNAs are associated with biological functions promoting cell-survival such as metabolism, cell communication, signal transduction and unexpectedly cell phenotype changes (e.g., development, morphogenesis or cell differentiation) (Fig. 5f and Supplementary Data 3). This observation highlights that, at the translational level, while a stress response associated with cell cycle arrest and cell death is observed, pro-survival-associated pathways are concomitantly upregulated. Therefore, we specifically searched for genes that may contribute to cell survival. To this end, we set up a list of genes promoting cell survival and tumor progression including genes associated with either mutations or copy number alterations in CRC, or associated with negative regulators of apoptosis and positive regulators of cell cycle functions. Interestingly, more mRNAs encoding survival proteins were found in the translationally upregulated mRNA category than in the translationally downregulated one, both in 10 µM and 50 µM 5-FU conditions (10 µM, 27 up vs. 18 down; 50 µM: 38 vs 30, Supplementary Data 4). Indeed, the cellular functions of the translationally upregulated mRNAs survival genes were mainly negative regulators of apoptosis, positive regulators of cell cycle and cell signaling factors (Supplementary Data 5). Altogether, these data indicate that 5-FU treatment results in an unexpected increase in translation of mRNAs encoding proteins with cell survival functions, which could contribute to the observed drug tolerance of a subset of cells.

Discussion
In this study, we reveal that the pyrimidine analogue 5-FU is incorporated into ribosomes in vitro and in vivo, including in human tumors. We used a LC-HRMS method that we recently developed in order to quantitate the level of incorporation of 5-FUrd into a defined RNA molecule. This approach allowed us to demonstrate that 5-FUrd is incorporated into ribosome at significant levels, showing that cells can tolerate the production of non-natural ribosomes. This finding was unexpected because ribosome assembly and maturation require multiple RNA-RNA and RNA-protein interactions and RNA folding that could be limited or inhibited by the presence of 5-FUrd, and because these steps are under stringent quality-control that induces the degradation of improperly folded and assembled rRNAs, as evidenced by the decrease in the level of the late pre-rRNA species that we report in this study. It can be anticipated that 5-FUrd would be enriched only in rRNA regions of the mature ribosomes where its presence does not significantly inhibit rRNA maturation and folding. As a result, cytoplasmic functional ribosomes contained up to 15 molecules of 5-FUrd per ribosome, a number likely underestimated since only a fraction of the ribosome population was renewed within the time frame of our experiment. We were able to observe 5-FUrd in RNA of 2 of 5 human CRC samples, highlighting that 5-FU incorporation into tumor ribosomes can occur in patients. We ruled out that the lack of detection of 5-FUrd in 3 of the samples was due the threshold of sensitivity of our assay. It is possible that incorporation takes place only in some tumors, depending on tumor cell metabolism or other molecular traits that remain to be determined. In addition, other parameters might influence 5-FU incorporation and detection such as the chemotherapy regimen or the delay between treatment and surgery. For these reasons, and because our analysis was performed on a small number of samples, we cannot at this stage extrapolate the frequency of incorporation. Most importantly, it remains to establish whether there is a link between 5-FU incorporation into tumor cell ribosomes, tumor response to therapy and disease outcome. While the addition of fluorine into RNA results in a non-natural modification, and could be anticipated as deleterious, we found that fluorinated ribosomes are functional as they engage in translation. However, their activity is altered and displays a selective ability to initiate mRNA translation according to the nature of its 5′UTR.
finding that fluorine appears to modify the functioning of rRNA is not unexpected since chemical modifications of rRNA including 2′-O-methylation and pseudouridylation were shown (i) to contribute to translational regulation and efficiency13–15,38, and (ii) to establish molecular interactions that cannot be provided by non-modified ones7,9. The effect we observe on translation initiation suggests that 5-FUrd incorporation is enriched at or close to regions of the ribosome that are critical for the interaction of the ribosome with mRNAs or translation factors, or in the functional domains of the ribosome, such as the A, P and E sites. Thus, if present in particular regions of the ribosome, even a limited number of 5-FUrd molecule could greatly influence the
Ribosomes were purified from HT116 cells treated with 10 μM 5-FU for 24 hours and their translational activity was evaluated using the hybrid in vitro translation assay. Translation efficiency was evaluated on luciferase reporter mRNA containing the 5′-untranslated region (5′-UTR) of the indicated gene. CDS = coding sequence. Values are units of Renilla luciferase activity normalized against the untreated (NT) condition. e, Evaluation on capped mRNA containing the 5′UTR of actin, GAPDH, IGF-1R and c-Myc genes. d Evaluation on uncapped mRNA containing the IRES element from the cricket paralysis virus (CrPV) and the encephalomyocarditis virus (EMCV). Data are expressed as mean ± s.d. of independent experiments (n = 3). e IRES activity of IGF-1R 5′UTR and deletions was evaluated by transfection of a bicistronic reporter vector represented on top of the figure. IRES activity is monitored as the ratio of firefly luciferase (Fluc) activity over Renilla luciferase (Rluc) activity. HT116 cells were transfected for 24 hours and then treated for 24 hours with either 10 μM or 50 μM 5-FU. Data are expressed as mean ± s.d. of independent experiments (n = 4). f Translation initiation activity of IGF-1R 5′UTR and deletions and of GAPDH 5′UTR was evaluated by co-transfection of a reporter vector represented on top of the figure with a renilla reporter vector for normalization. Translation activity is monitored as the ratio of firefly luciferase (Fluc) activity over Renilla luciferase (Rluc) activity. HT116 cells were transfected for 24 hours and then treated for 24 hours with either 10 μM or 50 μM 5-FU. Data are expressed as mean ± s.d. of independent experiments (n = 4). Results of unpaired two-tailed t test are indicated as nonsignificant (ns) p > 0.05 (*), p < 0.01 (**) and p < 0.001 (***) and p < 0.0001 (****). Source data are provided as a Source Data file.

ribozyme behavior. For instance, in the case of IRES-dependent translation, 5-FUrd may be selectively enriched in rRNA regions close to RPS25, RPL40 or RACK1 proteins that were shown to participate in IRES-dependent translation39–41, or within 18S rRNA regions proposed to support IRES translation, such as the expansion segment ES742 or the 959-964 region, which was proposed to interact with IGF-1R mRNA43. New tools will be required to finely map the location of 5-FUrd within rRNA and improve our understanding of its impact on ribosome functioning at the atomic level.

We previously described a major translational reprogramming induced by 5-FU in colorectal cancer cells, that we have linked to a miRNA-based mechanism44. Here we describe the 5-FU induced translational reprogramming by RNA-Seq-based polypeptide profiling. Upon 5-FU exposure, the majority of translationally altered mRNAs are upregulated, indicating that 5-FU allows maintenance or increased translation of these mRNAs. The fluorination of rRNA that we describe herein represents an additional mechanism by which 5-FU contributes to translational reprogramming of treated cells44. In addition to miRNA-based translation regulation, IRES-dependent translation appears as another mechanism, which 5-FU can modulate. IRES-dedicated genome-wide assay will be necessary to evaluate which IRES elements might be sensitive to 5-FU44. It is likely that other mechanisms are involved, such as 5-FUrd incorporation into mRNAs and tRNAs.

We determined that the 5-FU altered translational machinery contributes to maintaining the expression level of the IGF-1R gene, thus promoting cell survival. This suggests that the cytotoxic efficiency of 5-FU may be improved if fluorinated ribosome production is prevented, an approach that could be effectively tested using the recently developed ribosome biogenesis inhibitors, for which anti-cancer activities are being unveiled45–47. Drug tolerance is a critical phase as it represents a window of opportunity for genomic and non-genomic events to take place and provides cells with a drug-resistant phenotype. We show that sustained IGF-1R synthesis is a significant factor for cell survival upon 5-FU treatment. Surprisingly, our data indicate that 5-FU sensitized cells to IGF-1/IGF-1R pathway, as 5-FU treated cells were more sensitive to IGF-1 or IGF-1R inhibitors than untreated ones. It is not clear whether this is directly related to changes in translational regulation, nevertheless, it suggests that targeting the IGF-1/IGF-1R pathway may improve 5-FU efficacy. This data also supports that the IGF-1/IGF-1R pathway might contribute to drug tolerance. In addition, several mRNAs coding for proteins carrying anti-apoptotic functions, metabolic functions and cellular differentiation functions were translationally upregulated in 5-FU treated cells, and may contribute to cellular treatment escape. Indeed, metabolic adaptation and phenotypic plasticity, have been associated with cellular drug tolerance, and the ability of a small number of cells to survive drug pressure in a quiescent or slow-proliferative state4,48. Our data further support that translation control could represent a non-genomic mechanism contributing to drug tolerance, and will require single-cell analysis49,50. 5-FU is the most widely used chemotherapy, and there is a high demand for improving its efficacy. Our data highlight the potential benefits of understanding drug-tolerance mechanisms in response to 5-FU, which has so far not been fully described. In addition, while our study focused on a base analogue incorporated into RNA, other compounds binding to RNAs such as platin derivatives or any drug that might interfere with RNA metabolism should now also be considered as modifiers of ribosome structure and activity51, and may also contribute to altering translational regulation in treated cells. Altogether, our study extends the spectrum of gene expression mechanisms that help cells survive a drug challenge, by adding translational regulation to epigenetics, stress response, metabolism adaptation and stemness or dormancy phenotypes1,2,4,42–54. These findings also reveal that exposure to drugs can result in the production of “man-made” biological complexes, the functioning of which cannot be anticipated, and that require further studies to fully comprehend drug response and propose new therapeutic strategies.

Methods

Cell lines, cell culture, and 5-FU treatment. Cells were maintained in Dulbecco Minimum Essential Medium—GlutaMax (Invitrogen) supplemented with 10% foetal bovine serum (FBS) at 37 °C with 5% CO2. The following cell lines were obtained from ATCC: HT116 (ATCC CCL-247), MDA-MB-231 (ATCC HTB-26), BT20 (ATCC HTB-38), HCT116 (ATCC C226) and Panc1 (ATCC CRL-1469) and MiaPaCa (ATCC CRL-1420). The following cell lines were obtained from the authors: ISRECO1 (Cajot, J. F., et al. Cancer Res. (1997) 57, 2593–2597). The following cell lines were authenticated by 21 PCR-single-locus-technology (Eurofins, Ebersberg, Germany): HCT116, HT29, MDA-MB-231, MiaPaCa, Panc1. ISRECO1 cell line was not authenticated by PCR-single-locus-technology as this cell line genetic pattern is not described in databases. Cells were routinely tested against mycoplasma infection.

Cells were plated 48 hours before 5-FU treatment. 5-FU was kindly provided by the Centre Léon Bérard (Lyon, FRANCE) and was purchased from Sanofi-Aventis. The stock solution was diluted immediately before use in DMEM.

Western Blot. Western blot was performed as previously reported55. Briefly, cells were counted and a defined number of cells were lysed in lysis buffer A (20 mM HEPES-KOH pH 7.2, 100 mM KC1, 1 mM DTT, 0.5 mM EDTA, 0.5% NP40, 10% Glycerol) supplemented with protease inhibitor (Complete EDTA free, Roche). A volume corresponding to 100,000 cells was loaded on a polyacrylamide gel, and
proteins were subjected to SDS-PAGE, and blotted onto poly-vinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). Membranes were blocked in Tris-buffered saline solution containing 0.05% Tween 20 and 5% nonfat milk and incubated with primary antibodies: mouse monoclonal antibodies against GAPDH [6C5] (AM4300, Invitrogen) and rabbit monoclonal antibodies against IGF-1R (#9750, Cell Signalling) and actin (ab179467, Abcam) and anti-Histone H3 (ab1791, Abcam) diluted at 1:2000. Horseradish peroxidase-conjugated secondary antibodies Antirabbit IgG, HRP-linked Antibody (#7074, Cell Signalling Technologies) and Antimouse IgG, HRP-linked Antibody (#7076, Cell Signalling Technologies) diluted at 1:5000 were used for the detection of immunoreactive proteins by chemiluminescence (Clarity Western ECL Substrate, BioRad). Imaging and densitometric measurements of the bands were performed using Image lab software (BioRad).
**Fig. 4 IGF-1R contributes to survival and recovery of 5-FU treated CRC cells.**

- **a**: HCT116 cells were treated with 10 µM or 50 µM 5-FU for 24 h or 48 h or untreated (NT). Translation efficiency of actin, GAPDH and IGF-1R mRNAs. Each mRNA was quantified from cytoplasmic and polysomal fractions. Translation efficiency are shown as the ratio of polysomal mRNA over the cytoplasmic mRNA. Each dot represents an individual biological sample measured in duplicate and data are expressed as mean ± s.d of independent experiments (n = 3).
- **b, c**: HCT116 cells were treated with 10 µM 5-FU for 24 h or 48 h or untreated (NT). Cells were counted and an equivalent number of cells were loaded in each well. IGF-1R, Actin, GAPDH and H3 proteins were detected by western blot. **b** Level of IGF-1R, Actin, GAPDH and H3 proteins quantified from the western blot in **b**. Signals of each protein was normalized to the untreated (NT) value. Each dot represents an individual biological sample and data are expressed as mean ± s.d of independent experiments (n = 2).
- **d, e**: HCT116 cells were treated with 10 µM 5-FU for 24 h or 48 h or NT, and not stimulated (No IGF-1) or stimulated with 5 or 10 ng/mL of IGF-1. Cell growth was monitored in real-time over 5 days. **d** Schematic representation of the experiment. **e** Growth rate measured over 72 h (day 6 to day 9). Each dot represents a technical replicate and data are expressed as mean ± s.d of independent experiments (n = 4). Results of unpaired two-tailed t test are indicated as nonsignificant (ns) p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****). Source data are provided as a Source Data file.

**Fig. 5 Alteration of specific mRNA translation by 5-FU.** HCT116 cells were untreated (NT) or treated with 10 µM or 50 µM 5-FU for 24 h and mRNA translation was assessed by RNA-sequencing of polyme-associated mRNAs. **a-b**: mRNA association with polysomes was compared between 10 µM (**a**) or 50 µM (**b**) 5-FU treated and untreated cells, and significance was tested using a two-sided Wald test from DESeq2 R package. Adjustments for multiple comparisons were made using Benjamini and Hochberg correction method. P values for individual mRNA are available in Supplementary Data 2. The data show all analyzed mRNAs, mRNAs whose polysomal level either increased (red) or decreased (blue) by more than twofold between untreated and 5-FU treated cells are colored (p(adj) < 0.05). The threshold +/+ = 1 log2(FC) is indicated by vertical lines. The number of significantly altered mRNAs is indicated on top of the graphs. **c** Same datasets as in (**a**) and (**b**). Venn diagrams showing the comparison of translationally upregulated and downregulated mRNAs in 10 µM and 50 µM 5-FU treated cells. **d** Gene ontology analysis of all translationally altered mRNAs upon treatment with 50 µM 5-FU. The data show enriched GO terms for biological processes category (GO-Term-BP) and KEGG pathways. Analysis performed using gprofiler23. **e-f**: Significantly enriched GO terms were clustered in sub-groups according to GOslim2 classification and using the CateGOrizier webserver64. The pie charts summarize the list of functional sub-groups in order of decreasing importance respectively for translationally downregulated genes (e) and translationally upregulated genes (f). The colours were chosen by order of importance, and do not represent the same groups in (e) and (f). Source data are provided as a Source Data file.
Purification of cytoplasmic ribosomes. Except otherwise stated, cytoplasmic ribosome purification from cells was performed as previously described39,40. Briefly, cytoplasmic ribosomes were obtained by magnetic UCA cell lysate to stabilize tissue homogenizer (Ozyme) and centrifugation at 12,000 g for 10 min to pellet mitochondria. To purified ribosomes, cytoplasmic fractions were adjusted to 500 mM KC1 by adding an appropriate volume of 3 M KC1 and loaded onto a 1 M sucrose cushion in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 500 mM KC1 and 2 mM DTT, and centrifuged for 2 h at 240,000 g. The pellet containing the ribosomes was quickly washed with and resuspended in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, and 25 mM KC1.

For puromycin treatment, cells were fractionated as described above. The cytoplasmic ribosome fraction was loaded onto a 1 M sucrose cushion in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2 and 2 mM DTT, and centrifuged for 2 h at 240,000 g. The pellet containing the ribosomes was quickly washed and was resuspended in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, and 25 mM KC1.

Ribosome purification from polysomal fraction. Cells were seeded at 107 cells/15 cm dish and treated with 5-FU 48 h later as indicated. Cells were then incubated for 5 min with 25 µg/mL Emetin (Sigma) and washed twice with cold 1X PBS before harvesting. Cytosolic lysates were prepared as described above. 3 µg of cytoplasmic proteins was loaded onto a 10–40% sucrose gradient, sedimented by ultracentrifugation for 2 h at 240,000 g at 37 °C. Fractions were collected and absorbance profiles were generated at 245 nm using an ISCO UA-6 detector. Polysomal fractions were pooled and concentrated in an Amicon Ultra-15 unit with a 100 kDa cut-off. KC1 concentration was adjusted to 500 mM using a 4 M stock solution. The ribosome suspension was reduced to 600 µL with Amicon Ultra-15 filtration and loaded onto a 1 M sucrose cushion in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 500 mM KC1 and 2 mM DTT, and centrifuged for 2 h at 240,000 g. The pellet containing the ribosomes was quickly washed and was resuspended in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, and 25 mM KC1.

rRNA purification. For purified ribosomes, rRNA was extracted using the TriPure Reagent (Roche) according to the manufacturer’s instruction. Purified rRNAs were resuspended in water and quantified by spectrophotometry. For xenograft and clinical samples, rRNAs were purified as described previously44. Briefly, 15 µg of total RNA was denatured in 50% formamide at 70 °C for 10 min, and separated on a 0.8% low-melting agarose gel in 0.5X TAE buffer. 18 S and 28 S rRNA were gel-purified using the Nucleoclean Gel and PCR Clean-up kit and Ntab buffer (Macherey Nagel) according to the manufacturer’s instructions.

5-FU analysis by LC-HRMS. Purified rRNA (1 to 3 µg) was digested overnight at 37 °C with 270 units of Nuclease S1 (Promega) using the supplied buffer. Next, nucleotides were dephosphorylated by directly adding to the mix 5U of calf intestine phosphatase (New England Biolabs) in 100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, 0.025% Triton X-100. Digestion was carried on overnight at 37 °C. The digested mix was then stored at 4 °C. For puromycin treatment, cells were fractionated as described above. The cytoplasmic ribosome fraction was loaded onto a 1 M sucrose cushion in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 500 mM KC1 and 2 mM DTT, and centrifuged for 2 h at 240,000 g. The medium was then added to 1 M sucrose cushion in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, and 25 mM KC1.

Radial immunodiffusion analysis of cell extracts. NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-27847-8 | www.nature.com/naturecommunications

Bioinformatic analysis of translatome data. TranslomE libraries read quality were assessed using FastQC v0.11.9 (Babraham Institute, Cambridge, UK). Reads were filtered according to quality threshold Q5 and were trimmed of 4 and 2 bases at their start and end, respectively, using Cutadapt v3.27. With Cutadapt we set the minimal length of trimmed reads at 50 nucleotides: all trimmed reads shorter than 50 were removed from the analysis. High-quality reads were then aligned using STAR v2.7.5a45, on the Homo sapiens reference genome, version GRCh38.dna. prog. The nearest assembly release v103 was annotated with Gencode v39. Gene annotation file. Quantification of mapped reads was performed using HTSeq-count v0.13.559, using the following parameters: strand = reverse, mode = intersection-nonempty, typeexpr = exon, id_attributes = gene_id, additional attributes = gene_name, and ignoring chimeric reads. Statistical differential analyses were performed between the control and each 5-FU conditions (10 or 50 µM) using the Wald test from DESeq2 R package60. For each dataset, the read counts were filtered with a minimum of 1 count per million per biological sample after size factors estimation (method RLE for Relative Log Expression normalisation), and then dispersion was estimated (using DESeq2). P value adjustment that corrects for multiple discovery to lower the risk of false negatives. Statistical differential analyses were performed with Benjamini and Hochberg61. Genes with corrected p values below 0.05 were kept. Gene identifications were performed with biomaRt R package62. Functional annotations were performed with pGorR63 using a gSCS threshold at < 0.80, and according to the Go terms of Survival genes was performed using the "functional annotation clustering" tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8, using default parameters84.

Immunofluorescence analysis. Cells were grown on glass coverslips, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) before permeabilization with 0.5% Triton X-100 in PBS. Bafilomycin, Dykerin and Nucleolin were detected using the anti-f-BRL rabbit polyclonal antibody (ab8521, Abcam) diluted at 1:2,000, anti-DKC1 rabbit polyclonal (sc-48794, Santa Cruz Biotechnology) diluted at 1:500 and anti-NCL mouse monoclonal antibody (AE2 (ab13541, Abcam) at 1:4,000. Secondary Antibody, Alexa Fluor 488 (A-21428, Thermofisher Scientific), Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11008, Thermofisher Scientific), Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (A-21422, Thermofisher Scientific), and Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (A-21428, Thermofisher Scientific) were used at 1:1,000. Coverslips were mounted using the Fluoromount G mounting medium (EMS). Images were acquired on a Zeiss LSM 780 confocal microscope using a 63X Plan Apochromat immersion objective (NA 1.4), as a Z-stack (voxel size: 0.0634 x 0.0634 x 0.3153 µm). Final images were prepared by maximum intensity projection to display all nucleoli, and fluorescent signal was analyzed using the ZEN Black software. As immobilized stages of actinomycin D treated cells was adjusted to correct for NCL dispersion. Images were cropped using ImageJ (https://imagej.net)86.
rRNAs were visualised by autoradiography using ImageQuant TL software (GEHealthCare) on a Typhoon PhosphorImager, (GE HealthCare). Isotope signal was normalised to ethidium bromide signal for 24 S and 18 S rRNA bands, and quantified using ImageJ (build bsd6864e55 - https://imagej.net).

Preparation of mRNA-associated polysomes. This was performed as described in32. Briefly, cells were seeded at 10^5 cells/15 cm dish and treated with 5-FU 48 h later as indicated. Cells were then incubated for 5 min with 25 μg/mL emetin (Sigma) and washed twice with cold 1X PBS before harvesting. Cytosolic lysates were prepared as described above. Three milligrams of ribosomal proteins were added onto a 10–40 % sucrose gradient, sedimented by ultracentrifugation for 2 h at 240,000 g at 4°C on a SW-41 rotor (Beckman). Fractions were collected and absorbance profiles were generated at 245 nm using an ISCO UA-6 detector. Fractions corresponding to the second half of the polysomes (heaviest polysomes) were pooled and RNAs were extracted with TriPure Reagent as described by the manufacturer (Roche).

Quantitative RT-PCR. Two hundred and fifty nanogram of total RNA were reverse transcribed using the M-MLV RT kit and random primers (Invitrogen), according to the manufacturer’s instructions. Quantitative real-time PCR (qPCR) was carried out using the Light cycle 480 II real-time PCR thermocycler (Roche). Expression of mRNAs was quantified using LightCycler 480 SYBR Green 1 Master Mix (Roche). The primers were obtained by oligonucleotide synthesis (Eurogentec) and are described in Supplementary Table 2.

Xenograft tumour model. Mice were 7 weeks old female Hsd: Athymic Nude-Foxn1nu (Envigo). 1.5×10^6 HCT116 cells were subcutaneously injected into nude mice flank (n = 5). At day 14, tumours had reached an average of 160 mm^3. Mice received three injections (days 14, 18, and 22) with either vehicle (n = 2) or 5-FU (50 mg/kg, n = 3). 4 h after the last treatment, mice were sacrificed and tumours were collected for qRT-PCR analysis. Total RNA was isolated from the TRI REAGENT protocol (SIGMA T9424) according to the manufacturer’s instructions. RNA sequencing data of translatome analysis generated in this study are available at the NCBI Gene Expression Omnibus database under accession numberGSE178839. Datasets were normalized to the untreated condition (NT).

Cell growth and viability assays. HCT116 were seeded onto 96-well plates at 3,000 cells/well. 48 h after seeding, cells were treated with 5 µM 5-FU alone or in combination with 5 µM NVP-AEW541 (Sigma-Aldrich), or with DMSO as a control, and for an additional 72 h with 5 µM NVP-AEW541 alone, and cell viability was evaluated using MTT assay (Cell Titer Aquous One Solution Cell Proliferation Assay, Promega) according to the manufacturer’s protocol. Cell growth was monitored in real-time using the xCELLigence technology (ACEA Biosciences), based on electric impedance generated by cells attached to the well. Signals were normalised against the time obtained with IGF-1 (5 or 10 ng/mL (Peprotech)) treatment. The growth rate was calculated over 72 h as the slope under the curve.

Data availability. RNA sequencing data of translatome analysis detailed above, statistical analysis was performed using the Prism software (version 7.0, GraphPad). A two-tailed unpaired student t-test was used for evaluating significance. IF, FISH and TEM experiments were performed at least 3 times, and images are representative of several fields observed for each experiment.

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Competing interests
The authors declare no competing interests.

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