Regulation of let-7 microRNAs by a two-step turnover pathway is of critical pathophysiological significance in humans

Rohit Nalawade[1], Tamoghna Chowdhury[1], and Saibal Chatterjee[1*]

Affiliation:
[1]Laboratory of RNA Molecular Biology, Indian Institute of Science, Bangalore, India

*****Correspondence to: saibal@iisc.ac.in

Abstract

MicroRNAs (miRNAs) are critical regulators of diverse developmental and physiological processes in animals, and their dysregulation has been linked to various disorders and diseases. Not only multiple regulatory mechanisms acting at different levels of human miRNA biogenesis determine miRNA abundance and function, but a few recently identified factors by facilitating miRNA turnover also make critical contributions. We demonstrate that the ribonuclease XRN2, whose worm ortholog had previously been shown to actively degrade let-7 family of miRNAs, can degrade the mature forms of certain let-7 family members in multiple human cancer cell lines, without affecting their precursors. The XRN2-mediated turnover of let-7 has patho-physiological significance as XRN2 depletion results in a reduction in the expression of a number of oncogenes, and diminishes the proliferative and metastatic potential of cancer cells. The clinical relevance of these observations is also verified in tumour transcriptomics data from public RNA-sequencing datasets, where we observe that higher *XRN2* mRNA expression is inversely correlated with the levels of mature let-7 miRNAs and associated with poor survival in hepatocellular carcinoma, lung adenocarcinoma, and glioblastoma. We also demonstrate that miRNA turnover is a step-wise process, where a miRNA is released from the grasp of Argonaute before its degradation. This yet unidentified 'miRNA releasing factor' is proteinaceous in nature and its activity is kinetically linked with XRN2-mediated turnover of miRNAs. Our analyses of the patient-derived transcriptomics data also show that XRN2, via its regulation of let-7, affects pathways related to cellular proliferation, development, and signalling in a consistent manner across epithelial and glial cell lineages. Collectively, our studies suggest an important role of XRN2 in regulating cancer physiology through degradation of the let-7 family of miRNAs.

Introduction

miRNAs function as critical regulators of diverse developmental and physiological processes in animals[1]. Extensive studies on miRNA abundance and function have demonstrated a consistent link between dysregulation of miRNAs and various diseases, highlighting the importance of robust regulation of miRNA activity[2]. Many miRNAs exhibit tissue- and/or stage-specific expression patterns[2, 3] and dramatic changes in the abundance of a number of miRNAs during different developmental stages of animals have also been reported[4]. Global kinetic studies of miRNA metabolism have revealed that regardless of high biogenesis, several miRNAs exhibit reasonably low steady-state levels[5,6]. It indicated that additional determinants, other than the factors of biosynthesis, play critical roles for the establishment of cellular miRNA homeostasis[5,6].

A substantial amount of knowledge has been acquired on the different steps of miRNA biogenesis and their regulation, but much less is known about the miRNA turnover pathways and the constituent molecular machineries[7]. Active turnover of miRNAs in animals was first reported in *Caenorhabditis elegans* (*C. elegans*), where 5'-3' exoribonuclease XRN-2 was demonstrated to mediate the degradation of several miRNAs[8]. Later, the paralogous protein, XRN-1, was also described as a 'miRNase' in worms[9,10]. In mammalian cells, a number of nucleases have been described to act on a given miRNA or a handful of miRNAs. XRN1 has been implicated in the turnover of miR-382 in HEK293 cells[11], and polynucleotide phosphorylase degrades miR-221, miR-222, miR-106b in human melanoma cells[12]. Tudor-SN was demonstrated to target several functional mature miRNAs, including miR-31 and miR-29b, which control specific mRNAs encoding cell cycle regulatory proteins[13]. Interestingly, Argonaute1 (Ago1)-bound miRNAs in *Drosophila* are known to be tailed by terminal nucleotidyl transferase[14] and trimmed by the 3' – 5' exoribonuclease Nibbler[15] upon binding an exogenously introduced target that harbours extensively complementary binding-sites for the given miRNA[14,15]. Notably, very few unusual extensively complementary endogenous targets have also been reported that lead to the 3'-end trimming-mediated destabilization of the cognate miRNA upon their interaction[16]. The terminal uridyl transferases TUT4 and TUT7 are known to participate in tailing of miRNAs across species from *C. elegans* to humans[17]. A recent work using HEK293T cells has identified a machinery consisting of TUTs and DIS3L2, which could execute the decay of a subset of Argonaute (AGO)-bound mature miRNAs that have exposed 3′ ends[18]. Very recently, two simultaneous reports have substantially accentuated our understanding on the mechanisms of target-directed miRNA degradation (TDMD), where it was described that pairing of RISC/AGO-loaded miRNA to certain unusual highly complementary targets triggers ZSWIM8 ubiquitin ligase-mediated degradation of AGO, rendering the miRNA susceptible to decay by unknown nuclease(s)[19,20]. Perturbation of ZSWIM8 led to the accumulation of several miRNAs in different mammalian cells and several other systems (worms and flies). Intriguingly, half-lives of AGO proteins are much longer than majority of the miRNAs[5,6], which suggested that ZSWIM8-mediated TDMD may not be the predominant miRNA turnover mechanism, rather, it is dedicated towards miRNAs that interact with unusual highly complementary targets. Thus, it is quite apparent that identification of yet unknown 'miRNases', followed by understanding of the pathways in which they are constituents, and their mechanistic details warrant further investigation.

Notably, although, knowledge about miRNA metabolism emanated from the worm system have been found to be largely conserved, but the role of the human ortholog of the worm 'miRNase'-XRN-2 remains unclear. Here, we explore the capacity of human XRN2 as a 'miRNase', but we decided to focus on the fundamentally important let-7 family of miRNAs that are known to regulate important target mRNAs in multiple tissues, whose perturbation leads to different disease states[2]. We demonstrate that XRN2

specifically acts on the mature forms of most of the let-7 family members. Rapid and efficient depletion of XRN2 leads to the accumulation of AGO-bound let-7 members that in turn downregulate their cognate targets, including proto-oncogenes. Depletion of XRN2 in different cancer cells affect their cellular physiology by reversing critical cancer-parameters, including epithelial-to-mesenchymal transition. Our *ex vivo* biochemical assays indicate that XRN2 mediated degradation of mature let-7 miRNAs happens upon 'release' of miRNAs from AGO by a proteinaceous factor without affecting AGO integrity, and these two steps are kinetically linked. We also demonstrate that our cell line-based observations are of critical significance as they correlate with the clinical data of cancer patients from public transcriptomics datasets. Here, our analyses partially explain the multifarious roles of XRN2 in cancer through its likely participation in miRNA turnover, with the observation that XRN2 influences multiple pathways related to cellular proliferation, development, and signalling, especially related to extracellular matrix (ECM) development, and that these influences are mostly consistent across both epithelial and glial lineages. These analyses also partially explain previous observations of elevated *XRN2* mRNA expression being associated with worse survival in lung cancer patients, and extend such observations into the more expansive Cancer Genome Atlas (TCGA) datasets for lung adenocarcinoma, hepatocellular carcinoma, and glioblastoma. Our analyses also suggest that for most of the let-7 family members, *XRN2* demonstrates a stronger inverse relationship with them than some of the recently identified factors implicated in miRNA turnover, at an RNA level. Collectively, our study reveals that human let-7 miRNAs are regulated by a two-step turnover pathway, wherein, XRN2 plays the role of a 'miRNase' in various tissues.

Results

XRN2 affects only the mature forms of human let-7 family members

In human HEK293T cells, XRN1 has been implicated in the decay of miR-382, whereas depletion of the paralogous XRN2 had no significant effect[11]. The authors performed shRNA-mediated knockdown of *XRN1* and *XRN2*, but did not demonstrate the knockdown efficiencies at the protein level. Additionally, substantial knockdown of *XRN2* was not achieved in these studies[11,21], which could be insufficient to exert an effect on all the pathways XRN2 is involved in. Therefore, prior to investigating the role of XRN2 in miRNA degradation in human cells, we optimized a rapid and efficient shRNA-mediated specific knockdown of XRN2 using a lentivirus-based approach (Supplementary Text 1, Figure S1).

By means of turnover, XRN-2 in *C. elegans* modulates the mature forms of different members of the let-7 family, which play important roles in development. Dysregulation of their homologues also perturbs development and leads to many disease states in vertebrates[2]. Therefore, we began by examining the level of human let-7a miRNA, an identical homolog of *C. elegans let-7*, in control and XRN2 knockdown cells. In HEK293T cells, where the level of endogenous let-7a is very low, XRN2 depletion did not affect its level (Figure 1A). A possible reason for this could be that XRN2-mediated turnover might not be a predominant factor in maintaining its abundance. Contrastingly, ~3-fold accumulation in the level of mature let-7a was observed in XRN2-depleted A549 cells (Figure 1A).

In animals, let-7 miRNAs are known to exhibit different levels of abundance in different tissues, which could be determined by different regulatory forces[22]. Thus, turnover mediated regulation of specific miRNA levels might be more important in certain tissues, whereas regulated biogenesis might be more critical in others. Accordingly, we selected additional cell lines from different cell lineages, which are known to express adequately detectable levels of let-7 family members[23]. We incorporated Glioblastoma cell lines LN229 and U87-MG, Hepatocellular carcinoma cell lines HUH7 and HEPG2, Breast cancer cell lines MDA-MB-231 and MCF-7, Lung cancer cell line A549 and Cervical cancer cell line HeLa in our study. XRN2-depleted lung (A549), hepatic (HUH7, HePG2), and neuronal (LN229, U87-MG) cells showed increase in the levels of their mature let-7a (Figure 1A, S2A). Conversely, let-7a miRNA did not accumulate upon XRN2 depletion in any of the breast or cervical cancer cell lines we examined (Figure S2A). This indicated that XRN2 might be involved in the determination of the levels of mature let-7 miRNAs in multiple, but not all human cancer cell lines.

Furthermore, we checked the effect of XRN2 depletion on other members of the let-7 family. TaqMan analyses revealed that XRN2 depletion affected many members, but not all. As demonstrated through

Northern probing, TaqMan analysis also showed 2-3 folds accumulation of mature let-7a, while other members of let-7 family including let-7c, -7e, -7g, and miR-98 were also upregulated to different extents upon XRN2 depletion in A549, HUH7 and U87-MG cells (Figure 1B). However, let-7b, -7d, -7f, and -7i did not show any significant upregulation in the levels of their mature forms. These data suggested that XRN2 can specifically affect certain let-7 family members in a cell or tissue specific manner.

Additionally, we reckoned that being a ribonuclease, XRN2 could affect miRNA levels either by degradation of the mature forms or by regulating the processing of the corresponding precursor molecules during biogenesis. To answer this query, we determined the levels of the precursor RNAs of let-7a and let-7g, whose mature forms are most accumulated upon XRN2 knockdown. RT-qPCR analyses did not show any significant upregulation in the levels of the pri-let-7a and pri-let-7g (Figure 1C, S2B). The relative levels of pre-let-7a and pre-let-7g remained unchanged in the XRN2-depleted samples, in comparison to the control samples (Figure 1D, S2C). Accumulation of mature let-7a and let-7g without any appreciable change in the levels of any of their precursor forms indicated that XRN2 acts specifically on mature let-7 miRNAs leading to their degradation in A549, HUH7, and U87-MG cells.

Accumulated miRNAs are functionally active: multiple validated targets of let-7 family members are affected upon XRN2 depletion in multiple cancer cell lines

Some of the let-7 family members in humans are known to act as tumour suppressors, and they get dysregulated in different cancers[24]. The tumour suppression ability of let-7 miRNAs is attributed to the fact that they target several important genes including *RAS*, *HMGA2*, *MYC*, *CYCD1* (Cyclin D1), and *CDC25A*[22,25], which are potent oncogenes in humans. We anticipated that if the accumulated let-7 miRNAs in XRN2-depleted cells would be rendered non-functional, the levels of their target mRNAs should remain unaffected. Therefore, we compared the levels of the validated target mRNAs of let-7 family members in control and XRN2-depleted A549, HUH7, and U87-MG cells. We noted a considerable reduction in the mRNA levels of the aforementioned targets in the experimental cells (Figure 2A, S2D), which clearly suggested that the accumulated let-7 family members are functionally active.

To augment our observations derived from cell culture-based studies, we investigated if the mRNA levels of the validated let-7 targets *KRAS*, *NRAS*, *MYC*, *HMGA2* and *CDC25A* were also affected in patients displaying high expression of *XRN2* mRNA in a public transcriptomics dataset for hepatocellular carcinoma (TCGA-HPCC). Each of these mRNAs was elevated in patients having higher than median *XRN2* expression as compared to patients with lower than median expression of *XRN2* (Figure 2C). These observations indeed corroborated with our results obtained from the aforementioned XRN2-depleted cell lines. Having observed this dysregulation of the oncogenic let-7 targets, we then investigated the

relationship of let-7 family members with *XRN2* mRNA levels in the same TCGA-HPCC dataset. We found that let-7a, -7c, -7f and -7g were inversely correlated with *XRN2* (Figure 2D, 2F), and indeed displayed reduced abundance in case of high *XRN2* (Figure 2E). Notably, here the status of let-7a, -7c, and -7g exhibited equivalence with our cell line-derived observations. We also noted similar observations in the datasets for lung adenocarcinoma (TCGA-LUAD) and glioblastoma (TCGA-GBM) (Figure S3A-D). Interestingly, let-7d and let-7e showed stronger inverse relationship with *XRN2* in GBM than in HPCC or LUAD (Figure S3B). However, let-7b was not inversely related with *XRN2* in GBM as it had been in HPCC or LUAD. These data indicated that the specificity of XRN2-mediated regulatory activities vary in different clinical tissue samples, similar to the way it varied in the above-described defined cancer cell lines.

Notably, a perfect let-7a-target mRNA can also be a target for the other members of the let-7 family. But the magnitude of regulatory effect of a given member would be dependent on its abundance, coherence with the target expression, as well as its subcellular localization; thus making a given target mRNA relatively more specific to a particular family member. We observed that let-7b and let-7i do not show any significant changes in their levels upon XRN2 knockdown in the examined cell lines, and let-7i was also not negatively correlated with *XRN2 mRNA* in any of the TCGA-HPCC, TCGA-LUAD or TCGA-GBM datasets (Figures 1B, 2D, 2E, S3C, S3D). To confirm that the unaffected levels of let-7b and let-7i were indeed manifested functionally, we checked the levels of known let-7b and let-7i target mRNAs, namely, *KLK6* (let-7i target)[26], *PLK1*, and *CCNB2* (let-7b targets)[27]. As expected, levels of these mRNAs were not affected upon XRN2 depletion (Figures 2B, S2E). *KLK6* mRNA levels were additionally also not affected in the clinical tumour sample data for hepatocellular carcinoma, glioblastoma, or lung adenocarcinoma (Figures 2C, S3A, S3B), further strengthening our observations regarding the specificity of the regulatory activity of XRN2 on miRNAs *in vivo*.

Effects of XRN2 depletion on cancer cells

Dysregulated XRN2 expression is a common motif in several types of cancers, with *XRN2* mRNA expression being increased in tumour tissues of brain, cervix, lung, head and neck, colon, prostate, and testis, as compared to normal tissues[28]. Increased *XRN2* mRNA expression has been observed to be associated with increased risks of developing lung cancer as well as increased severity and poor prognoses in lung cancer[21]. Decreased expression of miRNAs of the let-7 family have also been correlated with poor survival of patients suffering from multiple types of cancers[29], let-7a[30] and let-7d[31] have been observed to show a reduction in their levels in human lung cancer and head-and-neck squamous cell carcinoma (HNSCC) tumours, respectively. As our results demonstrated XRN2-mediated turnover of let-7 miRNAs in cancer cells from lung adenocarcinoma, hepatocellular carcinoma, and glioblastoma, we performed an independent Kaplan-Meier survival analysis on the heterogeneous TCGA datasets for glioblastoma

(TCGA-GBM), hepatocellular carcinoma (TCGA-HPCC) and lung adenocarcinoma (TCGA-LUAD) to explore any possible link with XRN2 (Figure 3A). Indeed, these analyses further verified that elevated levels of *XRN2* mRNA were associated with worsened survival outcomes in these patient-derived datasets.

let-7 miRNAs are known to repress proliferation and facilitate differentiation by affecting multiple critical pathways in human cells[2,24] . Previous studies in human lung adenocarcinoma and ovarian cancer cell lines have reported that overexpression of certain let-7 family members results in anti-proliferative effects and reduced survival of cancer cells[24,32]. Our observations on the levels of mature let-7 miRNAs upon XRN2 knockdown, with a concomitant effect on the levels of crucial target-oncogenes, prompted us to check if XRN2 depletion can lead to the reversal of tumour phenotypes in these cancer cell lines. Notably, cell migration is a crucial process in cancer progression that allows cancer cells to metastasize to different tissues through the circulatory system. To test whether XRN2 depletion can affect the migration of these cancer cells, we performed a wound healing assay, which allowed us to study directional migration of cancer cells *in vitro*[33]. We observed that the migration of A549 and HUH7 cells undergoing XRN2 knockdown was significantly reduced as compared to the control cells (Figure 3B, C, data not shown). We further performed clonogenic assays, which examines the ability of a single cancer cell to undergo multiple divisions and grow into a colony. This assay performed with the aforementioned two cell lines revealed diminished colony formation ability in XRN2-depleted cells as compared to the control cells (Figure 3D, E, data not shown). Of note, cell migration assay couldn't be performed with U87 cells as they start forming spheroids before reaching confluence. Also, survival of U87 cells was drastically affected upon XRN2 depletion, which made them unsuitable for performing clonogenic assays that require prolonged maintenance. Overall, these results demonstrated that the changes in miRNA levels upon XRN2 depletion are indeed physiologically significant as they affect the proliferation and migration of the cancer cells.

Notably, certain let-7 family members have already been shown to function as vital regulators of the Epithelial-to-Mesenchymal transition (EMT), which is considered to be a prerequisite for acquisition of malignant phenotypes like metastasis by epithelial cancer cells[33]. Accordingly, we checked the expression levels of E-cadherin (*CDH1*), which is primarily expressed in epithelial cells, and is known to undergo a reduction in its expression during EMT, and the mesenchymal marker Vimentin (*VIM*), which shows increase in its level during EMT. As expected, depletion of XRN2 in A549 cells, drastically reduced the *VIM* mRNA expression, while the expression of *CDH1* mRNA showed a significant upregulation. Another mesenchymal marker, *SNAI1*, also showed a decrease in its expression level in XRN2-depleted cells (Figure 3F). mRNA levels of certain important matrix metalloproteases such as MMP2 and MMP9, which are vital for metastasis of cancer cells, were also found significantly downregulated in XRN2-depleted cells (Figure 3G). Notably, similar results were also obtained with HUH7 and U87 cells (data not shown). Together,

these results indicated that XRN2 depletion impedes EMT transition and metastatic progression in cancer cells.

Turnover of let-7 miRNAs is kinetically linked to the release of miRNAs bound to AGO2

miRNA biogenesis is completed with the generation of an AGO-loaded mature miRNA, as a constituent of a macromolecular complex called miRNA-induced silencing complex (miRISC)[1]. We wanted to check how XRN2 depletion affects the mature miRNAs bound to the AGO proteins. Accordingly, we immunoprecipitated the endogenous AGO2 from either control or XRN2-depleted A549 cells to check the levels of let-7a miRNA associated with immunoprecipitated AGO2. A significantly increased signal was recorded from AGO2 immunoprecipitated from the XRN2-depleted cells (Figure 4A). Similar observations were made upon immunoprecipitation of AGO2 from HUH7 and U87 cells (data not shown). Furthermore, we checked the levels of other let-7 sisters associated with immunoprecipitated AGO2 from A549 cells. Here, we observed that amongst all the let-7 miRNAs, let-7a, -7c, -7e, -7g, and miR-98 showed the highest accumulation in immunoprecipitated AGO2 from XRN2-depleted cells compared to control (Figure 4B), and that further indicated to a dislocation in the cascade of events leading to turnover of these miRNAs. We were intrigued by this observation to check whether those miRNAs undergo a 'release step' from AGO before degradation, as it has been reported elsewhere[8], and how XRN2 may affect this process. Accordingly, we performed an *ex vivo* 'miRNA release assay', as has been described earlier[8], employing immunoprecipitated endogenous AGO2 from A549 cells (Figure 4C). Immunoprecipitated bead-bound AGO2, upon incubation with control lysate and a further recovery, showed a significant loss of let-7a signal, when compared to the signal obtained from the bead-bound AGO2 incubated with buffer (Figure 4D top panel). No miRNA could be detected in the RNA recovered from the supernatant fraction of the release assay, where bead-bound immunoprecipitated AGO2 was treated with control lysate (Figure 4D middle panel). However, the assay that employed XRN2-depleted cell lysate for incubation with bead-bound immunoprecipitated AGO2, more let-7a could be detected in the recovered bead-bound immunoprecipitated AGO2, post treatment (Figure 4D top panel). A substantial amount of let-7a could also be detected from the RNA extracted from the supernatant fraction of the same reaction (Figure 4D middle panel), which clearly demonstrated that those miRNAs are not associated with AGO2, but still not degraded. This data suggested that a diminished level of XRN2 in knockdown samples might be good enough to perform the 'release activity' but unable to degrade the released let-7a miRNA. Alternatively, XRN2 is kinetically linked to the process of let-7a release from AGO2, performed by a dedicated 'miRNA release factor' that is not present in molar excess in comparison to XRN2. However, since, no XRN2 protein could be detected with immunoprecipitated AGO2 (data not shown), it could be suggested that either the AGO2-XRN2 interaction is highly transient or there is a yet unidentified 'miRNA release factor' that facilitates miRNA release from AGO2 for its degradation. Notably, subjecting the above-mentioned control

cell lysate with protein denaturing conditions resulted in a complete loss of its 'miRNA releasing activity', which suggested a proteinaceous nature of the 'miRNA release factor' (Figure S4A).

As treatment with total cell lysate resulted in an efficient release of let-7a from immunoprecipitated AGO2, we checked whether other let-7 sisters got similarly released from the AGO2 protein. We observed that let-7a and let-7g underwent release to the extent of 85-90%, while the other sisters showed 60-80% release from AGO2 (Figure 4E).

Comparative analyses of protein mediated release of miRNAs from Human AGO2 with miRNA release mediated by highly complementary target RNAs

Spanning over a decade, several reports have highlighted an alternative pathway for miRNA degradation, which primarily relies on release or tailing-and-trimming/ trimming-mediated destabilization of miRNAs from miRISC upon binding to target RNAs that possess extensive complementarity with the miRNAs [34,35,14,16]. Accordingly, *in vitro*-reconstituted AGO2-guide RNA complexes have been demonstrated to unload the guide RNA strand upon incubation with high molar excess of perfectly complementary target RNA sequences[34]. We therefore sought to perform a comparative study between the release of the AGO2-bound miRNAs mediated by the extensively complementary target RNAs, and the above-mentioned total cell lysate devoid of any nucleic acid due to treatment with micrococcal nuclease. We began by monitoring the release of let-7a from endogenous AGO2 in the presence of increasing concentration of an *in vitro*-transcribed target RNA, perfectly complementary to let-7a (Figure 5A-D, S4B). We observed release of around 90% AGO2-bound let-7a upon incubation with ~150 folds molar excess of the target RNA at 37°C for 30 minutes (Figure 5D). Interestingly, very little or no miRNA was released from AGO2, when the reaction was performed with equimolar concentration of target RNA (Figure 5C). Contrastingly, ~80% of endogenous AGO2-bound let-7a was released upon incubation with total lysate (75 µg), where amount of the lysate was in equivalence with that of the immunoprecipitated AGO2 employed in the 'miRNA release assay' (Figure 5C, 5E). Notably, with ~5% immunoprecipitation efficiency, the immunoprecipitated AGO2 employed in each miRNA release assay reaction was equivalent to ~75 µg of total lysate (Figure S4C), and from which ~20 fmol of let-7a could be extracted (Figure S4D). Upon incubation with 150 µg of total lysate, the release of let-7a was further facilitated to the extent of ~90% (Figure 5E). Similarly, let-7d, which was only 60% released upon treatment with 75 µg total lysate, showed a greater magnitude of release upon treatment with higher concentrations of total lysate (Figure 5E). These results clearly demonstrated that the release of miRNA from miRISC that could be achieved at a high molar excess of target RNA, could also be achieved with total protein lysate, but at a physiologically relevant concentration. Of note, in the aforementioned target-mediated release assay, we also measured the magnitude of release for other members of the let-7 family, for whom the employed let-7a-target RNA was only partially complementary.

Indeed, the incompletely complementary target failed to efficiently release let-7i (4 mismatches) or let-7d (2 mismatches) from the endogenous AGO2, even when it was present at 150-fold molar excess (Figure 5D, S4E). Together, our data suggest that protein-mediated miRNA release from miRISC is an important mechanism for miRNA metabolism in humans.

Interestingly, we observed that let-7b, let-7d and let-7i, unlike other let-7 family members, were not released to a substantial amount from the endogenous AGO2 upon exposure to the total lysate (Figure 4E). This could possibly be due to the following three reasons: (i) their release is partially dependent on target RNAs, (ii) the factor(s) responsible for their release from the specific miRISCs were partially inactive in the employed total lysate due to extraction conditions, or (iii) the composition of the respective miRISCs housing let-7b, let-7d and let-7i were such that they were resistant to the releasing activity, which might confer a greater stability and longer half-life to them. Indeed, by inhibiting RNA Pol II with α-amanitin, we could examine their relative stabilities, and they appeared to be way more stable than the other members of the let-7 family (Figure 5F, data not shown). This data suggested that the composition of a specific miRISC or a specific miRNP structure formed by a set of proteins and a given miRNA, may override a general turnover mechanism, and thus play an important role in determining the functionality of the given miRNA.

Analysis of clinical cancer transcriptomics data reveals the widespread impact of XRN2 on miRNA homeostasis and cellular pathways.

As previous reports have implicated the ZSWIM8 Ubiquitin ligase and Tudor-SN (*SND1*) as two crucial regulators of miRNA abundance in mammalian cells[13,19,20], we became interested to learn how these candidates affected the expression levels of the let-7 family members in comparison to *XRN2* in the aforementioned TCGA datasets. In this direction, we compared the fold changes in the mean levels of let-7 family miRNAs between high and low expression categories of *XRN2*, *SND1* and *ZSWIM8* mRNA in the TCGA-HPCC hepatocellular carcinoma clinical tumour sample dataset (Figure 6A). We chose to perform this comparison on a set of 100 samples, which displayed the greatest mean fold change for let-7a and let-7g between the low and high expression categories for *XRN2* mRNA (see Methods for selection procedure). In this subset, let-7a, -7b, -7c, -7f, -7g and miR-98 showed a stronger inverse relationship with *XRN2* mRNA than with those of the other two enzymes, which is further borne out by their increased accumulation in case of low *XRN2* mRNA levels than for the other enzymes considered (Figure 6B). We also observed similar results for the TCGA-GBM and TCGA-LUAD datasets with *XRN2* expression having the stronger inverse relationship with the majority of let-7 family members (Figure S5A, S5B), as was the case in the TCGA-HPCC dataset (Figure 6A). The above observations clearly suggested that amongst these three candidates, XRN2-mediated turnover might have the greatest impact on regulation of the levels of most of the cellular let-7 miRNAs.

Furthermore, since, let-7 family members have been adequately characterised to be involved in regulating several important cellular pathways[2], we went on to investigate if any other major cellular pathways, aside from those directly associated with cancers, were affected in relation to *XRN2* expression in the employed clinical tumour transcriptomics datasets. Gene Ontology (GO) terms are used to annotate genes with their functional roles in pathway databases such as the KEGG[36] and Reactome databases[37]. Therefore, we performed GO term enrichment analysis on genes, which are predicted or validated targets of let-7 family members (as per human TargetScan 7.2[25]). Among these genes, whose mRNAs were also positively correlated with *XRN2* mRNA in the relevant TCGA-HPCC dataset were selected for downstream analysis (Figure 6C). Given the roles of let-7 family members in regulating pluripotency, especially through the conserved LIN28B axis[22], it was unsurprising to find that one of the enriched terms was related to regulation of stem cell pluripotency, and other terms related to differentiation. Similarly, due to the known roles of let-7 family members in regulation of genes involved in controlling the cell cycle like *CDK6*, *CDK8*, *CDC25A* and *CYCLIN D*[22], we also found terms related to cell cycle processes and mitotic checkpoints to be enriched. We additionally observed that regulation of cellular senescence through the p53 and Ras-MAPK/RTK signalling pathways[38] were also enriched terms in the term enrichment analysis. Since, let-7 family members are also known to regulate the TGF-β receptor TGFBR1, as well as STAT3 and PI3K-AKT[39], terms related to TGF-β and PDGF signalling were also enriched. Furthermore, it was interesting to observe that terms related to collagen biosynthesis, focal adhesion and syndecan interactions displayed enrichment as well. Notably, this could be because let-7a, let-7b and let-7g are known to regulate the expression of type 1 collagen genes[40]. Overall, outcomes of this analysis suggest a strong involvement of both XRN2 and let-7 family members in regulation of cell proliferation and differentiation, as well as cell and extracellular matrix (ECM) homeostasis.

Very similar GO terms were enriched in the outcome of a similar analysis performed on the TCGA-GBM dataset (Figure S5C). The outcome of the GO term enrichment analysis performed on the TCGA-LUAD dataset was less intriguing, since, both hepatocellular carcinoma and lung adenocarcinoma are cancers of epithelial tissue. Notably, we found that while some common terms related to MAPK signalling and cell cycle regulation were enriched, but the variety of enriched terms was much lower (data not shown). This could most probably be due to a higher sample heterogeneity in the TCGA-LUAD dataset as compared to the TCGA-HPCC and TCGA-GBM datasets, which might have allowed far fewer GO terms to reach the requisite significance threshold for representation.

It is interesting that we found very similar GO terms enriched in the TCGA-HPCC and TCGA-GBM datasets, as it suggests that these roles for XRN2 and let-7 family miRNAs are preserved across different

cell lineages, and further suggests far-reaching roles for XRN2 in various aspects of epithelial and glial development and homeostasis.

Discussion

Our results demonstrate that in human cancer cells, turnover of let-7 family of miRNAs is a layered process involving multiple steps (Figure 7), and it acts in conjunction with other regulatory forces to govern the functionality of miRNAs. We furnish the role of XRN2 as a critical regulator of miRNA abundance with a prominent impact on the physiology of cancer cells. Our findings suggest that the putative 'miRNA release factor' and XRN2-mediated miRNA decay might together constitute an important pathway to regulate the stability and function of let-7 members.

A previous study reported that XRN2 binds pre-miR-10a in a DICER-independent manner and accelerates the maturation of miR-10a in human lung cancer cells[21], but did not provide insights on the members of let-7 family. Here, we demonstrate that in a number of human cancer cell lines of lung, hepatic, and neuronal origin, XRN2 depletion led to the accumulation of a number of mature let-7 family members without any effect on their pri- or pre-miRNAs. It was also observed that XRN2 depletion led to a significant decrease in the levels of oncogenes that are validated let-7 targets in cancer cells. The clinical significance of our findings was reinforced by the analyses of public tumour transcriptomics data from patients with lung adenocarcinoma, hepatocellular carcinoma, and glioblastoma, especially considering the heterogeneity of the patient cohorts. The transcriptomics analyses demonstrated an appreciable inverse relationship between the levels of certain let-7 family members and *XRN2* mRNA in the clinical tumour samples, which agreed with our cell line-based observations. They also revealed that let-7 target genes positively correlating with *XRN2* mRNA expression are involved in multiple signalling pathways regulating cell proliferation and differentiation, as well as in biogenesis and maintenance of the extracellular matrix in both epithelial and neuroglial cell lineages. This partially reveals why elevated *XRN2* has previously been associated with worse survival outcomes in lung cancer patients, and also accentuates prior observations on overexpressed *XRN2* mRNA in spontaneously occurring lung tumours in mice and human tumour microarray data analysed through genome-wide association studies[28]. Our observations from analyses of the TCGA-LUAD, TCGA-HPCC and TCGA-GBM datasets also extend the association of high *XRN2* mRNA levels with worse survival to lung adenocarcinoma, hepatocellular carcinoma, and glioblastoma, respectively. Our motivation towards performing bioinformatic analyses employing the TCGA datasets was because of how they encompass samples from a large and diverse patient population for each cancer type and the corresponding dataset, while ensuring that all sequencing data acquisition and processing have been performed using uniform procedures. We expect that our observations, especially considering the large and heterogenous clinical population represented in the TCGA datasets, would advocate for wider applicability of the molecular signatures of XRN2 in determining cancer severity than what could be suggested from smaller or more limited datasets. With the revelation of a clear inverse

relationship between the levels of let-7 members and XRN2 expression, we found that for two other important factors implicated in miRNA turnover, such an inverse relationship with let-7 miRNA levels was either much weaker (e.g., *SND1* mRNA) or largely non-existent (e.g., *ZSWIM8* mRNA). These observations suggest that XRN2-mediated degradation could potentially be the 'housekeeping' mechanism for turnover of let-7 miRNAs in mammalian cells.

It has been previously described that exogenously introduced target RNAs having extensive complementarity to the cognate miRNAs can lead to target RNA–directed tailing and trimming of endogenous miRNAs, and influence miRNA stability and function[14]. Structural studies have demonstrated that the 5'-end of a miRNA remains anchored in a pocket in the MID domain of AGO, and the 3'-end is bound by the PAZ domain. The miRNA remains largely buried in a channel between the two lobes of AGO, except the bases of the miRNA-seed region, which remain partially exposed to the surface of the host protein. However, extensive base pairing of AGO2-bound miRNA with target RNAs disrupts the 3'-end anchoring and renders the 3′-end accessible for enzymatic activity[41]. Also, specific AGO-bound miRNAs that have their 3'-ends exposed, have been demonstrated to undergo an oligo-tailing, followed by degradation by DIS3L2[18]. Since, for most cellular AGO-bound miRNAs, their 3'-ends are protected by the AGO proteins, unless they encounter extensively complementary targets, it is improbable that DIS3L2 mediated miRNA turnover pathway could be the foremost channel for miRNA turnover inside the cell[18]. Our studies provide evidence that XRN2-mediated degradation of miRNAs is dependent on an upstream event of 'miRNA release' from miRISCs, carried out by a yet unidentified proteinaceous factor(s). Moreover, our results indicate that protein factor-mediated release of AGO-bound let-7 appears to be far more efficacious compared to complementary target RNA-mediated mechanism[34]. Additionally, this two-step turnover mechanism has the potential to prevent immediate degradation of an AGO-released miRNA, which might enable shuttling of some specific candidates to the yet uncharacterized non-canonical pathways that utilize mature miRNAs without any association with known components of miRNA metabolism like AGO, GW proteins, etc.[42,43], whereas, most of the released miRNAs might get delivered to the bona fide 'miRNases' for degradation.

Interestingly, the recently reported 'miRNase'-Tudor-SN co-immunoprecipitates with RISC subunits and appears to be a component of miRISC[11]. The intracellular signalling events marking an AGO-residing miRNA for termination of its function remain largely unknown, and it will be important to know the responsible factors and the mechanism that allows such miRNAs to travel from the grasp of AGO to Tudor-SN for degradation.

Downregulation of endogenous miRNAs has been shown to be induced by certain viral RNAs[44-47], as well as a few genome-encoded transcripts[16], which bear extensive complementarity to specific miRNAs. Recently, highly complementary target interactions were demonstrated to induce TDMD, independently of tailing and trimming events, by causing destabilization of AGO proteins through the ZSWIM8 ubiquitin ligase[19,20]. It has been proposed that ZSWIM8 directly recognizes the conformational changes induced in AGO upon extensive base-pairing of the resident-miRNA (miR-7) with a highly complementary target (Cyrano lncRNA), but the mechanism for this recognition remains to be understood. Since, it is well known that 3'-tailing often acts as an important signal for downstream processes[14,35], in its absence, it is quite possible that there might be yet unknown factor(s) that would facilitate recognition of the conformational changes elicited upon highly complementary miRNA-target interaction by ZSWIM8, in order to mediate TDMD. Additionally, the nucleases participating in the degradation of miRNAs, post their release from AGO undergoing proteolysis, needs to be identified. It is also noteworthy that the half-lives of AGO proteins are longer than most of the miRNAs they host[5,6], suggesting that miRNA release and degradation through destabilization of AGO by ZSWIM8 and related factors is unlikely to be the major pathway for miRNA turnover. Intriguingly, a highly complementary viral RNA element-mediated degradation of a host miRNA may be considered an efficient viral strategy to destabilize the host system. However, turnover of a miRNA due to highly complementary interaction with the cognate endogenous target, mediated by the destabilization of the AGO itself, would certainly pose an energy burden on the cellular system. Importantly, such unusual endogenous targets are not plentiful, and thus, TDMD through destabilization of AGO may have evolved as a regulatory measure under special circumstances for a specific set of miRNAs with exclusive functions. Contrary to TDMD, pairing of target mRNA with its cognate miRNA has been shown to exert a protective effect on miRNAs of specific families from cellular degradation in *C. elegans*[9]. Furthermore, target mediated protection of a few miRNAs was also reported to be crucial for controlling miRNA levels and their subcellular localization in human cells[48]. Also, in human cells, biogenesis of specific miRNAs was stimulated upon binding to their cognate target mRNAs[49]. Collectively, these reports indicate that probably there is no single principle and mechanism of turnover that determine the stability or vulnerability of miRNAs in different tissues and organisms.

let-7 members are amongst the highly conserved animal miRNAs that critically regulate expression of many genes governing diverse biological processes[1,2]. Interestingly, aside from their well-studied roles as tumour suppressors, let-7 family miRNAs are also important for mounting immune responses in vertebrates upon exposure to pathogens[50]. It is known to repress both the pro-inflammatory cytokine interleukin 6 (IL-6) and the anti-inflammatory cytokine IL-10 during *Salmonella* infection in murine phagocytic RAW264.7 cells, and thereby regulate early and late immune responses[50]. Interestingly, XRN2 depletion in RAW264.7 cells led to the upregulation of certain let-7 family members, and that in turn affected the levels of interleukins

(data not shown), and this clearly makes XRN2 a potential candidate for modulation of inflammatory responses during infection. Notably, many of the patients infected by SARS-CoV-2 have been reported to show uncontrolled elevation in cytokines with persistent acute inflammation. Failure of the cellular homeostatic machinery to appropriately downregulate these 'cytokine-storms' leads to a vicious cycle of accumulating inflammation and subsequent lethality of the condition[51]. Since, let-7 has been shown to be capable of targeting multiple interleukins, its regulation through XRN2 might play a vital role in such critical situations. Moreover, IL-6 is also implicated in regulating various hypothalamic functions and contributes to the activation of hypothalamo-hypophyseal-pituitary axis, and thus to overall metabolism in vertebrates[52,53]. Supported by our cell culture-based results employing neuronal cancer cells and patient samples of neuronal tissues (Fig. S2), it is possible that in the aforementioned neuro-endocrine tissues, let-7 plays a modulatory role on IL-6, and that in turn gets regulated by XRN2. Overall, these evidence and suggestions indicate that the regulation of let-7 through XRN2-mediated degradation might be conserved across the eukaryotic systems, and have widespread implications ranging from cancer physiology to host responses during infection. Further exploration of the functions of XRN2 and other components of the XRN2-mediated miRNA turnover pathway could shed new light on how these crucial regulators of the transcriptome themselves get regulated, and thus, may also provide opportunities for the discovery of new diagnostics and therapeutics.

Author Contributions

SC designed the research and experiments. RN and TC performed the experiments. Together, all the authors analyzed the results and wrote the manuscript.

Acknowledgments

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Figures and Legends

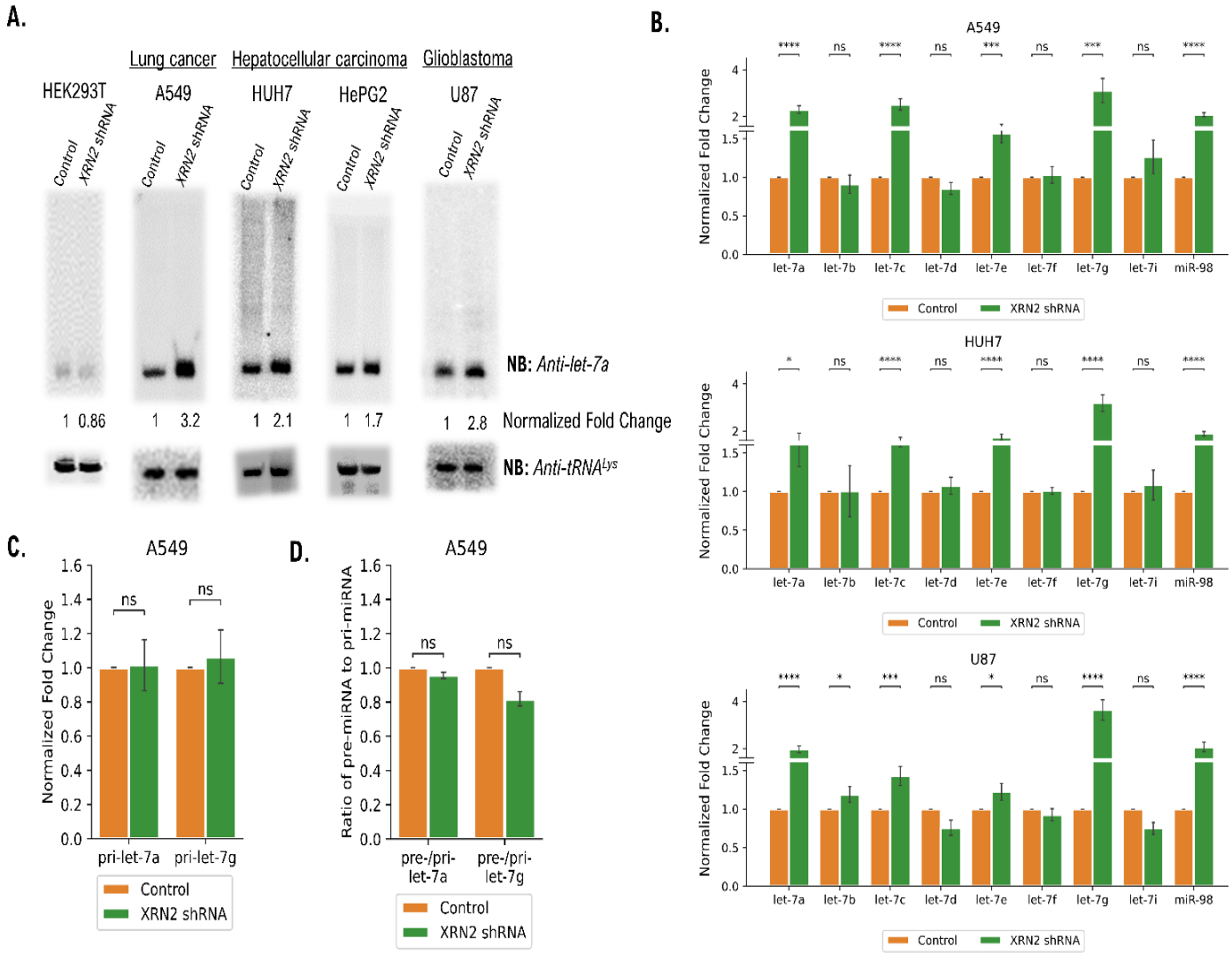

Figure 1 – Accumulation of specific let-7 family members in different human cancer cell lines upon depletion of XRN2.

A. XRN2 depletion leads to the accumulation of mature let-7a in A549, HUH7, HepG2, U87 cell lines, but not in HEK293T.

B. TaqMan analyses reveal increased abundance of the mature forms of different let-7 family members upon depletion of XRN2 in the indicated cell lines. RNU48 RNA served as the normalization control.

C. RT–qPCR (n = 4; mean ± SEM) analyses reveal no significant change at the level of primary RNAs for the indicated miRNAs in A549 cells.

D. RT-qPCR (n = 4; mean ± SEM) analyses depict no significant change in the relative levels of pre-miRNA transcripts for indicated miRNAs in A549 cells.

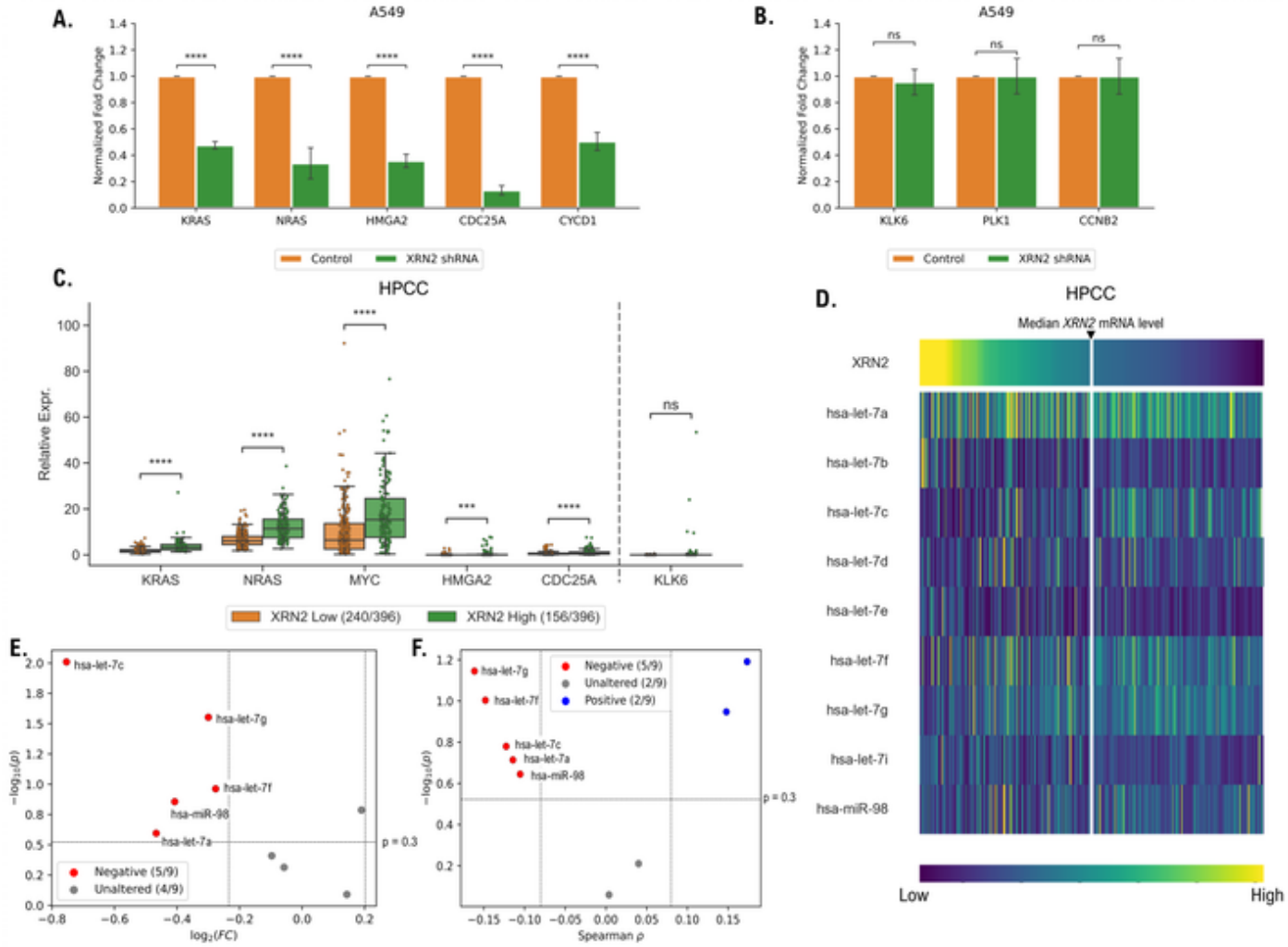

Figure 2 - XRN2 is linked to the expression of various oncogenes in cancer cell lines and patient tissues.

A. RT–qPCR (n = 4; mean ± SEM) analyses reveal reduction in let-7 target mRNAs, as indicated, upon XRN2 depletion in A549 cells. Fold changes were normalized to *ATP5G* mRNA. P-values are from paired Student's t-test.

B. Known let-7b and let-7i targets remain unaffected in XRN2-depleted A549 cells.

C. Relative mRNA expression levels (arbitrary units) of the indicated let-7 target genes in the high and low *XRN2* mRNA expression groups from the TCGA-HPCC RNA-Seq dataset of clinical hepatocellular carcinoma (HPCC) tumour samples. *KLK6*, a let-7i target that is unchanged upon XRN2 depletion in cell lines (B), is also not affected by different *XRN2* expression levels in the clinical tumour dataset. P-values are from Student's t-test with Bonferroni correction.

D. Expression patterns of let-7 family members in relation to *XRN2* mRNA levels in the TCGA-HPCC dataset. let-7a, -7c, -7f and -7g are inversely correlated with *XRN2.* Colour Gradient: Yellow – highest expression, Blue – lowest expression (relative to the minimum and maximum expression of the respective miRNA or mRNA). The split line through the heatmap denotes the median expression value of *XRN2* mRNA.

E. Volcano plot of significance of fold change (y-axis: $-\log_{10}(p)$) vs. mean fold change (x-axis: $\log_2(FC)$) of let-7 family members between high and low *XRN2* mRNA expression groups in the TCGA-HPCC dataset. Five out of nine let-7 sisters (let-7a, 7c, -7f, -7g and miR-98) are significantly negatively altered (top-left) in the high *XRN2* group, and are likely to be targeted by XRN2 in HPCC. Significance threshold was set at p = 0.3 to allow for the heterogeneity in the dataset, Student's t-test with Bonferroni correction.

F. Volcano plot of significance of correlation (y-axis: $-\log_{10}(p)$) vs. Spearman ρ correlation (x-axis: Spearman ρ) of let-7 family members with *XRN2* mRNA expression further confirms the inverse relationship of the five let-7 sisters (let-7a, 7c, -7f, -7g and miR-98) with *XRN2* mRNA, thus strengthening the conclusion that they are likely to be targeted by XRN2 in HPCC from (E). Significance was calculated similarly to (E).

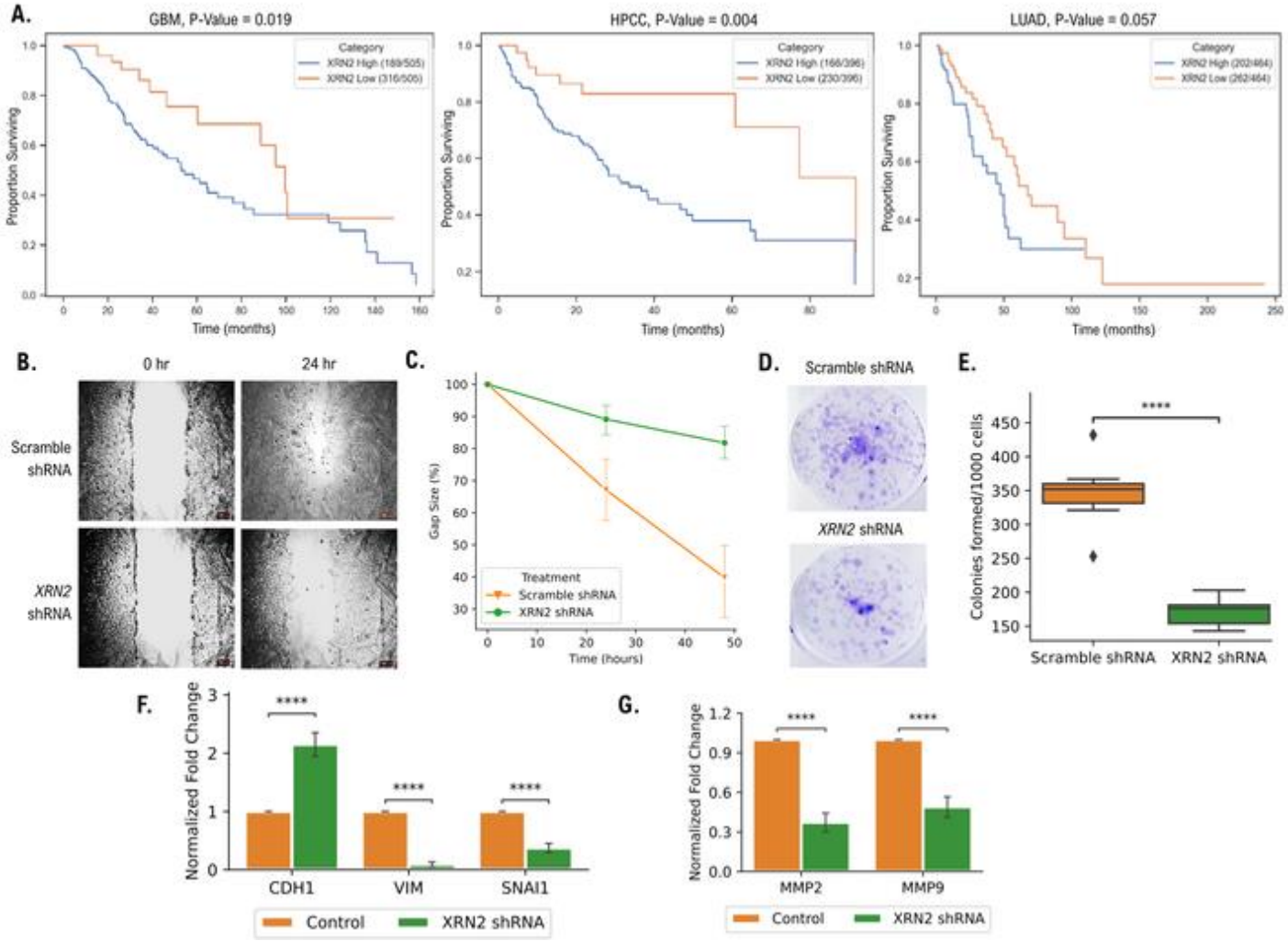

Figure 3 – High *XRN2* mRNA expression worsens survival of cancer patients, and its depletion affects cellular physiology of cancer cells.

A. Kaplan-Meier survival analyses indicate association of high *XRN2* mRNA levels with worsened survival outcomes in glioblastoma (TCGA-GBM), hepatocellular carcinoma (TCGA-HPCC), and lung adenocarcinoma (TCGA-LUAD). p-values are from Chi-Square test with d.f. = 2.

B. Wound-healing assay performed with A549 cells (n=4) transfected with *XRN2* shRNA depict reduced cell migration.

C. Graphical representation of the wound-healing assay reveals significant differences in the reduction of gap-size between the indicated samples.

D, E. Clonogenic assay with untreated and XRN2-depleted A549 cells (n=4), and corresponding box plot demonstrate the decrease in the number of colonies formed by XRN2-depleted cancer cells.

F. Upregulation in *CDH1* (E-cadherin) expression with simultaneous downregulation in *VIM* (Vimentin) and *Snai1* mRNA expression in XRN2-depleted A549 cells is suggestive of an increase in the epithelial signature over that of the mesenchymal. RT-qPCR (n = 4; mean ± SEM) results were normalized against *ATP5G* mRNA.

G. Downregulation in expression of mRNAs of the matrix metalloproteases, *MMP2* and *MMP9*, critically important in EMT, in *XRN2*-depleted A549 cells. RT-qPCR (n = 4; mean ± SEM) results were normalized against *ATP5G* mRNA.

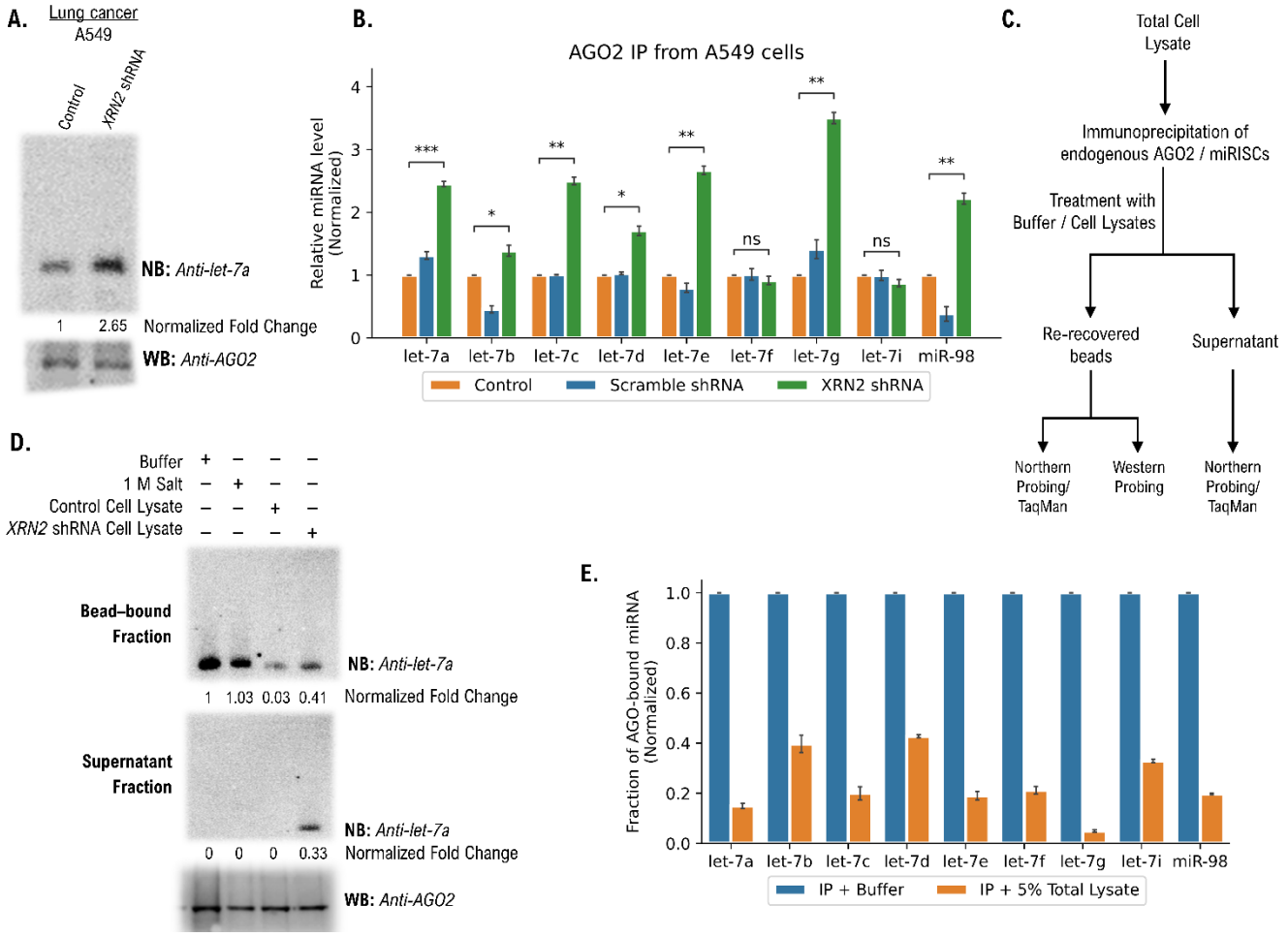

Figure 4 – Increased signal of let-7 miRNAs from AGO2 immunoprecipitated from XRN2-depleted cells.

A. Immunoprecipitation using anti-EIF2C2 (anti-AGO2) antibody from total cell lysate of the indicated samples reveal increased signal of let-7a in AGO2 of XRN2-depleted cells. One half of each immunoprecipitate was subjected to Northern probing or TaqMan analysis, the other half to anti-AGO2 Western probing and served as loading control.

B. TaqMan analyses (n = 3; mean ± SEM) reveal increased accumulation of several let-7 sisters in immunoprecipitated AGO2 from XRN2-depleted samples.

C. Schematic representation of miRNA release assay. Bead-bound immunoprecipitated AGO2/ miRISC were subjected to different treatments as indicated, and recovered back as bead and supernatant fractions. One half of each re-recovered bead fraction was subjected to Northern probing or TaqMan analysis, the other half to anti-AGO2 Western probing. Northern probing was performed for equivalent amount of RNA extracted from the supernatant fraction. Loss of signal from the re-recovered intact AGO2/ miRISC subjected to a specific treatment, compared to that of the buffer/high salt treatment, indicates release of AGO2/ miRISC-bound let-7a miRNA.

D. miRNA Release assay with immunoprecipitated AGO2 was performed with the indicated samples. Release of let-7a is diminished in the assay employing XRN2-depleted total lysate compared to the one with control lysate.

E. TaqMan analyses (n = 4; mean ± SEM) of the RNA isolated from re-recovered bead-bound fraction reveal differential release of different let-7 sisters from immunoprecipitated AGO2 subjected to control cell lysate.

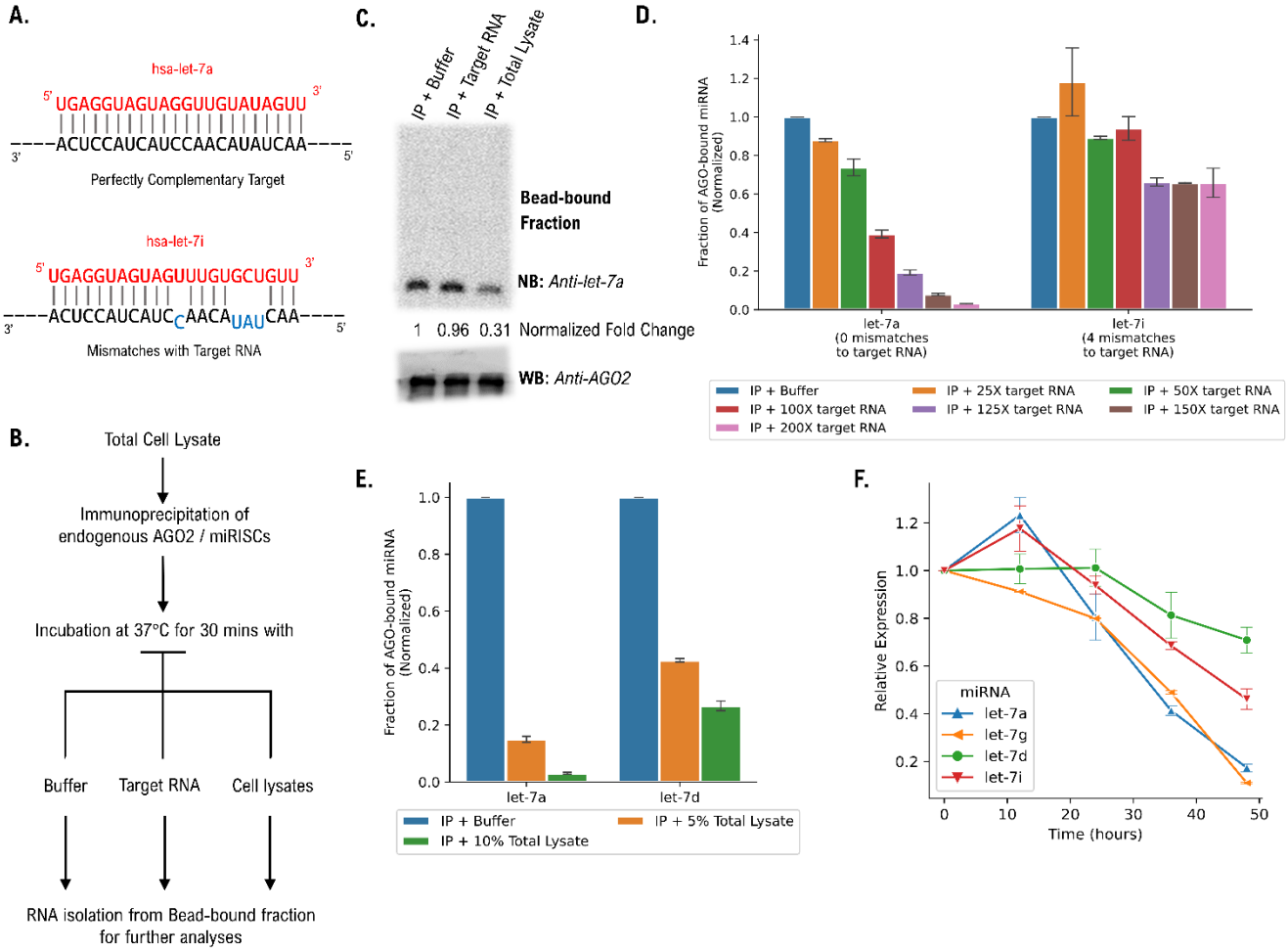

Figure 5 – Nucleic acid depleted total lysate releases let-7 miRNAs from miRISC more efficaciously than completely complementary RNA.

A. Schematic of *in vitro*-transcribed target RNA perfectly and imperfectly paired to let-7a and let-7i, respectively.

B. Schematic representation of miRNA release assay to check the release of let-7 miRNAs from endogenous AGO/ miRISC mediated by complementary target RNAs or total cell lysate.

C. Release of let-7a miRNA from immunoprecipitated AGO2 upon treatment with equimolar concentration of perfectly complementary target RNA or an amount of total cell lysate in equivalence with the employed AGO2. Post treatment, one half of the bead-bound AGO fractions were subjected to Northern probing (top) and the other halves were subjected to anti-AGO2 Western blotting acting as loading controls (bottom).

D. TaqMan analyses (n = 4; mean ± SEM) reveal differences in the magnitude of release of two let-7 miRNAs from immunoprecipitated AGO2 upon treatment with increasing concentrations of a target RNA (perfectly complementary to let-7a, but with 4 mismatches for let-7i).

E. TaqMan quantification (n = 4; mean ± SEM) of AGO2-associated let-7a and let-7d (re-recovered bead-bound fraction) upon treatment with different concentrations of total cell lysates. Let-7a is more amenable to release than let-7d.

F. Total RNA was isolated from cells harvested at 0, 12, 24, 36 and 48 hrs following α-amanitin (10 µg/ml) treatment, and TaqMan quantification performed for the indicated let-7 members that reveal differences in their relative stabilities.

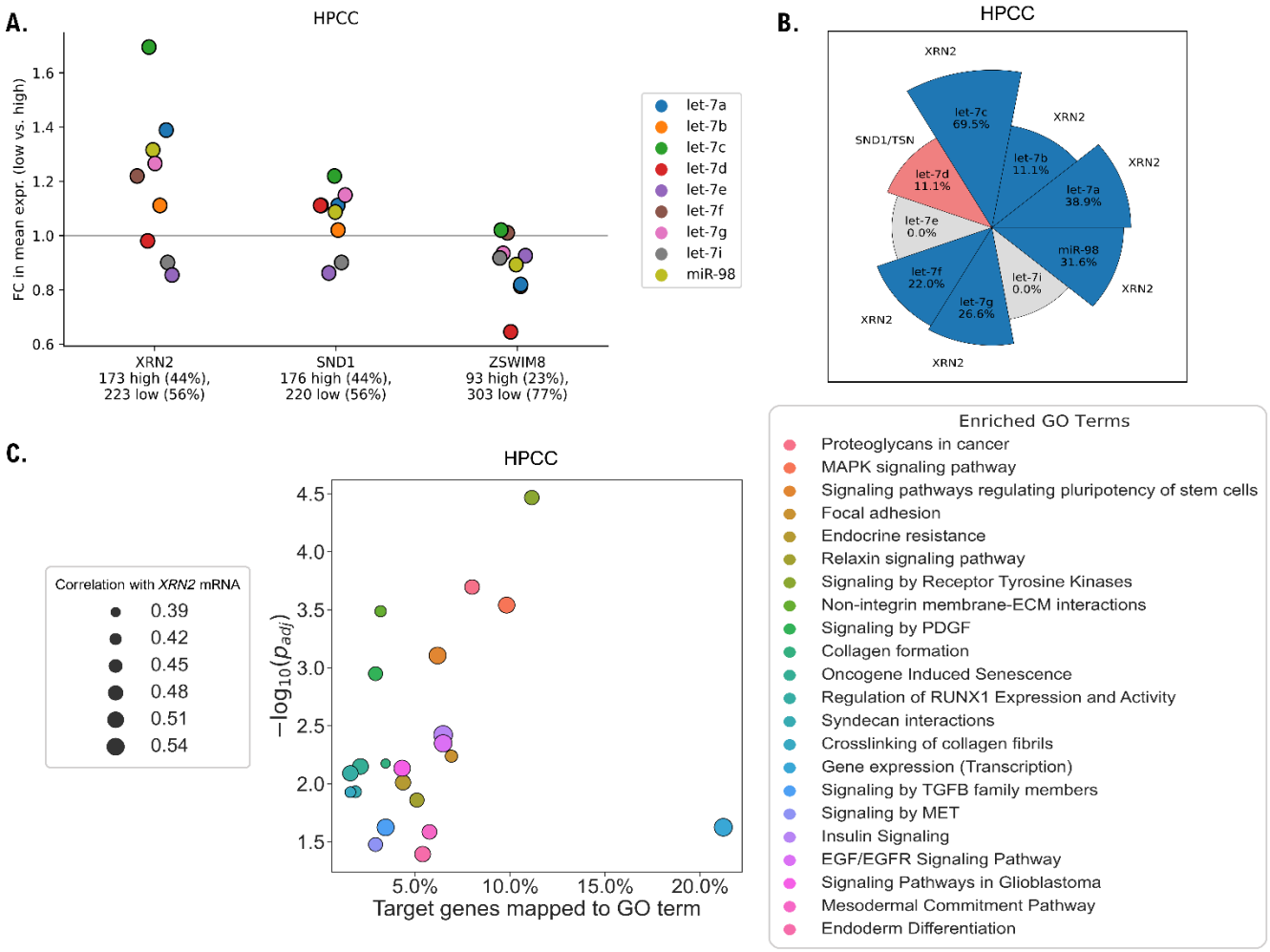

Figure 6 – Widespread impact of XRN2-mediated regulation of human let-7 miRNAs on various cellular processes they are implicated in.

A. Comparison of the mean fold change in expression of different let-7 family members between low and high expression groups for *XRN2*, *SND1* (Tudor-SN), and *ZSWIM8* mRNAs in a 100-patient subset of the TCGA-HPCC dataset (see Materials and Methods for details about the selection of the subset). In this subset, low *XRN2* expression is associated with larger increases in let-7a, -7b, -7c, -7f, -7g, and miR-98 levels in comparison to low expression of either *SND1* or *ZSWIM8*.

B. Relative accumulation of let-7 sisters due to low expression of *XRN2*, *SND1*, and *ZSWIM8* from (A). Six out of nine let-7 members are maximally accumulated in case of low *XRN2* over low *SND1* or *ZSWIM8*. Among these, let-7a and let-7c display the most accumulation for low *XRN2*, while let-7d is accumulated more for low *SND1*. let-7e and let-7i are not appreciably accumulated for low expression of any of the three enzymes considered.

C. Gene Ontology (GO) Term Enrichment analysis indicate the most enriched GO terms for the genes positively correlated with *XRN2* expression in the TCGA-HPCC dataset, include candidates from multiple signalling pathways as well as pathways related to ECM metabolism. “Signalling by Receptor Tyrosine Kinases” is the most significantly enriched GO term (y-axis: significance of enrichment), while “Gene expression (Transcription)” of both the most abundant and enriched GO term (x-axis: fraction of analysed genes associated with a GO term) is also strongly correlated with *XRN2* expression (depicted marker radii on the left box are proportional to the mean Spearman correlation of all the GO-term associated genes with *XRN2* expression). This observation is consistent with many let-7 targets being transcription factors. GO terms directly related to cancer or viral infection are not shown.

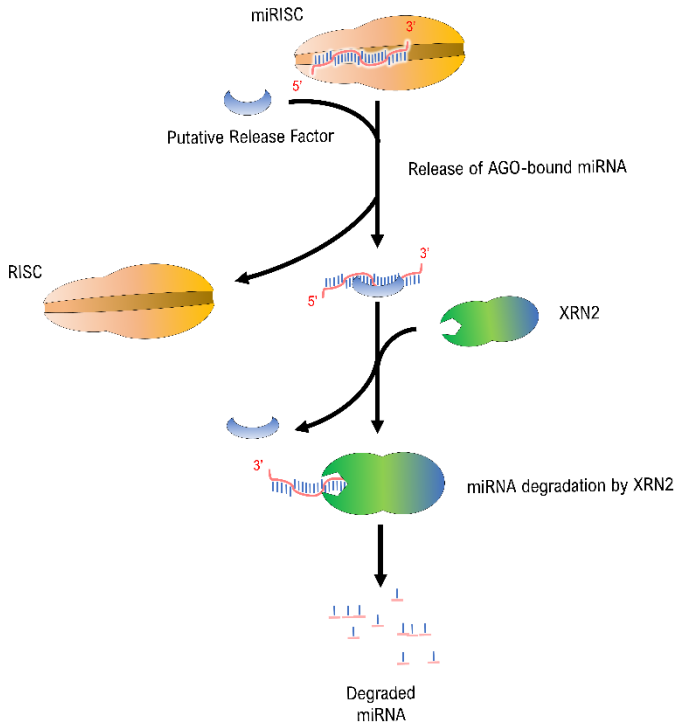

Figure 7 – A putative model for the XRN2-mediated miRNA turnover pathway.

A two-step miRNA turnover model, where XRN2 acts on the miRNA upon its release from the miRISC/ AGO by a yet unknown ‘miRNA release factor’.