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* Equal Contribution

MicroRNA-mediated mRNA Translation Activation in Quiescent Cells and Oocytes Involves Recruitment of a Nuclear microRNP

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MicroRNAs can promote translation of specific mRNAs in quiescent (G0) mammalian cells and immature *Xenopus laevis* oocytes. We report that microRNA-mediated upregulation of target mRNAs in oocytes is dependent on nuclear entry of the microRNA; cytoplasmically-injected microRNA repress target mRNAs. Components of the activation microRNP, AGO, FXR1 (FXR1-iso-a) and miR16 are present in the nucleus and cytoplasm. Importantly, microRNA target mRNAs for upregulation, Myt1, TNFα and a reporter bearing the TNFα AU-rich, microRNA target sequence, are associated with AGO in immature oocyte nuclei and AGO2 in G0 human nuclei, respectively. mRNAs that are repressed or lack target sites are not associated with nuclear AGO. Crosslinking-coupled immunopurification revealed greater association of AGO2 with FXR1 in the nucleus compared to cytoplasm. Consistently, overexpression of FXR1-iso-a rescues activation of cytoplasmically-injected RNAs and in low density, proliferating cells. These data indicate the importance of a compartmentalized AGO2-FXR1-iso-a complex for selective recruitment for microRNA-mediated upregulation.

MicroRNAs are small, non-coding RNAs that function as post-transcriptional regulators of gene expression upon recruitment into effector complexes (microRNA protein complexes or microRNPs), which contain a core Argonaute protein, AGO. MicroRNPs recognize the target mRNA, normally within its 3′-UTR, in a sequence specific manner and exert effects directed by the nature of the basepairing[3,7]. MicroRNAs have been primarily demonstrated to cause downregulation of mRNA expression[7]; however, under certain conditions, such as quiescence, distinct mRNAs demonstrate microRNA-mediated upregulated translation[17,22,26,31,42,47,52–56] or relief of repression by microRNAs[5,27,48].

Quiescence refers to the reversible, non-dividing G0 and G0-like states that dividing cells can enter for periods of time during which they exhibit specific gene expression[12]. Like G0 cells, the *Xenopus laevis* prophase I-arrested immature folliculated oocyte does not proliferate or replicate DNA and, as in some G0 cells, is maintained by cAMP/PKA signaling[6,43]. We previously demonstrated that a distinct microRNP comprising AGO2, microRNAs and a specific isoform of the Fragile-x-mental retardation related protein 1 (FXR1) family of proteins, called FXR1-iso-a, can elicit translation upregulation of minimal target reporters and of specific mRNA targets in quiescent mammalian cells[53–56] and in quiescent-like *Xenopus laevis* immature folliculated stage IV-VI oocytes[38]. Other groups revealed that specific microRNAs may activate translation of select transcripts including TNFα in quiescence and quiescent-like states[17,31,52,53].

We demonstrated that microRNA-dependent activation of the *Xenopus* Myt1 mRNA (via miR16 microRNA) is required in part to maintain the immature state. As in mammalian G0 cells, a *Xenopus* Argonaute (AGO) and FXR1 are required for translation upregulation by microRNAs in immature folliculated oocytes[38]. Argonaute slicer activity was reported to be absent in oocytes. An AGO protein, recognized by an antibody that detects the AGO PIWI domain is present in limiting amounts but not required for cleavage activity in the oocyte[35], suggesting that oocytes may harbor microRNAs to conduct cleavage-independent regulatory functions, such as translation activation.

Here, we report that a nuclear microRNP, comprising AGO2 and FXR1-iso-a, associates with specific mRNAs, leading to their upregulated expression in immature, folliculated oocytes and in human G0 cells. We show that microRNAs are required to be injected into folliculated oocyte germinal vesicles (GV) or nuclei to demonstrate

upregulated expression; cytoplasmic injections demonstrate repression of these mRNAs. The components of the activation complex for endogenous Myt1 mRNA—AGO, FXR1 and miR16[38]—are present in the nucleus of immature oocytes. Consistently, *Xenopus* AGO is associated with endogenous Myt1 mRNA in isolated oocyte nuclei, as is human AGO2 with TNFα mRNA or with a reporter bearing its AU-rich microRNA target sequence in G0 mammalian nuclei, but not in proliferating cell nuclei. *Xenopus* Cyclin A1[32] and mammalian Cyclin E mRNAs[34], which are repressed in immature oocytes and mammalian G0, are not recruited to the nuclear AGO/AGO2 complex, suggesting that recruitment to the nuclear complex contributes to selection of target mRNAs for microRNA-mediated activation. AGO2 and FXR1, previously reported to be present in mammalian cell nuclei[1,2,8,14,15,28,41,49,50,57,60], interact to a greater extent in the nuclei of oocytes and mammalian cells compared to the cytoplasm. Importantly, overexpression of FXR1-iso-a increases AGO levels and the activation complex in the cytoplasm and can rescue activation after cytoplasmic injection or in low density proliferating cells. These data indicate that selective recruitment of specific microRNAs and targets by a compartmentalized AGO2-FXR1-iso-a complex, present in the nucleus or upon overexpression, leads to activation of target mRNA expression.

Results

Translation upregulation is observed upon introduction of the microRNA and mRNA into the nucleus but not into the cytoplasm. Our previous results demonstrated that immature follicular *Xenopus laevis* oocytes showed activated expression of Myt1 mRNA injected into the nucleus in response to endogenous microRNA, miR16, and of CX, a Luciferase reporter bearing four artificial microRNA sites, injected as a DNA plasmid along with its corresponding synthetic microRNA, miRcxcr4, into the nucleus[38]. Fig. 1a shows that injection of capped CX reporter mRNA and miRcxcr4 into the oocyte cytoplasm does not yield translation activation, but instead exhibits modest (~ 2-fold) repression. The requirement of nuclear injection for activation of CX mRNA is specific for the corresponding microRNA and is not induced by a control microRNA (Fig. 1a). The cellular levels of the mRNA after nuclear versus cytoplasmic injections of the microRNA did not correlate with the translation outcome (Fig. 1b;[38]). Immunoprecipitation of AGO revealed more substantial association (25% compared to 4%) of the target reporter CX mRNA from nuclear-injected samples compared to cytoplasmically-injected samples (Fig. 1c). These results suggest that nuclear events enable the assembly of an appropriate mRNP for translation activation.

AGO is present in the nucleus in immature *Xenopus laevis* oocytes. We next examined the localization of factors expected to participate in activation in the nuclei and cytoplasm after enzymatically and manually defolliculating immature stage IV oocytes. The fractionation was verified by the presence of histone H4 in nuclei and tubulin in cytoplasm (Fig. 2a). Nuclear and cytoplasmic extracts were prepared in high salt buffer, then diluted to 100 mM salt followed by sonication, which enables release of AGO from heavy cytoplasmic complexes[38]. In all other figures, we avoided sonication to preserve the complex. Importantly we find that AGO is present in the nucleus (Fig. 2a). These data are consistent with a nuclear presence of the microRNP complex, previously reported for AGO2 and other AGOs in mammalian cells where the complex is required for functions in the nucleus[1,8,41,50,57,60]. Flag-tagged clones of *Xenopus* AGO[38] and hAGO2 expressed in rabbit reticulocyte lysate by *in vitro* translation and purified over a Flag column were detected by the Millipore AGO2 antibody (shown) or pan-AGO 2A8 antibody[38], validating the use of these antibodies for detection of *Xenopus* AGO (Fig. 2b). Additionally, immunoprecipitation of *Xenopus* AGO with the 2A8 AGO antibody (recognizes all mammalian AGOs but not PIWI[39]) co-precipitated a similar-sized band as detected by Western analysis with the Millipore AGO2 antibody[38]. Since 1) these oocytes were pre-defolliculated enzymatically and manually and 2) AGO was detected in isolated nuclei (that are not associated directly with follicles) and 3) AGO was detected by immunoprecipitation and Western analysis with two antibodies that recognize cloned *Xenopus* AGO (matching database NM_001093519 and similar to EU338243[38]) as well as human AGO2 (Fig. 2b), the AGO present in the nuclear compartment is potentially distinct from an AGO previously detected in the cytoplasm and primarily in the follicles[35].

An endogenous microRNA, miR16, is present in the oocyte nucleus. We examined the localization of mature miR16, which is required for translation activation of the Myt1 mRNA in immature oocytes and for maintenance of the immature state[38]. Stage IV oocytes were fractionated; the fractionation was verified by the presence of U6 snRNA in nuclei and 5.8S rRNA in cytoplasm (Fig. 2c). Endogenous miR16 is substantially present in the nucleus as detected by Northern analysis (Fig. 2c) and could be detected in the cytoplasm with a more sensitive splint ligation assay[38](Fig. 2d). As previously identified by splint ligation[38], the specific 5′ trimmed form of miR16, which activates Myt1 mRNA translation, is present in the nucleus and to some extent in the cytoplasm (Fig. 2d). These data suggest that some endogenous microRNAs, like miR16, have a nuclear phase, which is consistent with the presence of AGO in the nucleus.

Cytoplasmically-injected mRNAs are translationally activated upon introduction of their targeting microRNA into the nucleus. We noted in our previous study that microRNAs must be injected into the animal pole/GV or nuclear side of the oocyte for functional efficacy[38], consistent with the nuclear presence of miR16 (Fig. 2c–d) to activate Myt1 mRNA. We investigated whether nuclear entry of a microRNA, mimicking the nuclear presence of miR16 implicated in activation of its target, Myt1[38] (Fig. 2c–d), would be sufficient to induce microRNA-stimulated upregulation of its corresponding target site-bearing reporter. *In vitro* transcribed reporters were injected into the cytoplasm in this experiment while the microRNA was injected into either the cytoplasm or the nucleus. A control microRNA was tested in parallel. As observed in Fig. 3a, reporter expression was stimulated only when the microRNA was injected into the nucleus, whereas the control microRNA had no effect. The reporter RNA levels did not correlate with the translation outcome and could not account for the expression level changes (Fig. 3b). Neither decreasing nor increasing mRNA or microRNA concentrations could induce activation after cytoplasmic injection[38]. We had previously demonstrated that the microRNA injected into the nucleus emerges into the cytoplasm after 1hr post-injection[38]. These results are consistent with the nuclear presence of endogenous AGO and miR16, which were previously demonstrated to activate endogenous Myt1 mRNA expression[38] and suggest that nuclear entry of the exogenous microRNA enables targeting of the corresponding target mRNA for microRNA-mediated activation in immature oocytes.

The endogenous target mRNA of miR16, Myt1 mRNA, associates with AGO in the oocyte nucleus. Nuclear localization of microRNAs and AGO (Fig. 2) has been observed in mammalian cells, suggesting a nuclear phase for the microRNP complex[1,8,41,50,57,60]. Many mRNAs have been demonstrated to associate with RNA-binding proteins in the nucleus[20,29,32], targeting the RNAs to their appropriate fates in the cytoplasm; examples include Fragile X proteins like FXR1[2,14,15,28,49]. We investigated whether target mRNAs for activation are uploaded into microRNPs in the nucleus, distinguishing them from target mRNAs for repression that are recruited cytoplasmically to the microRNP for downregulation[57]. Consistently,

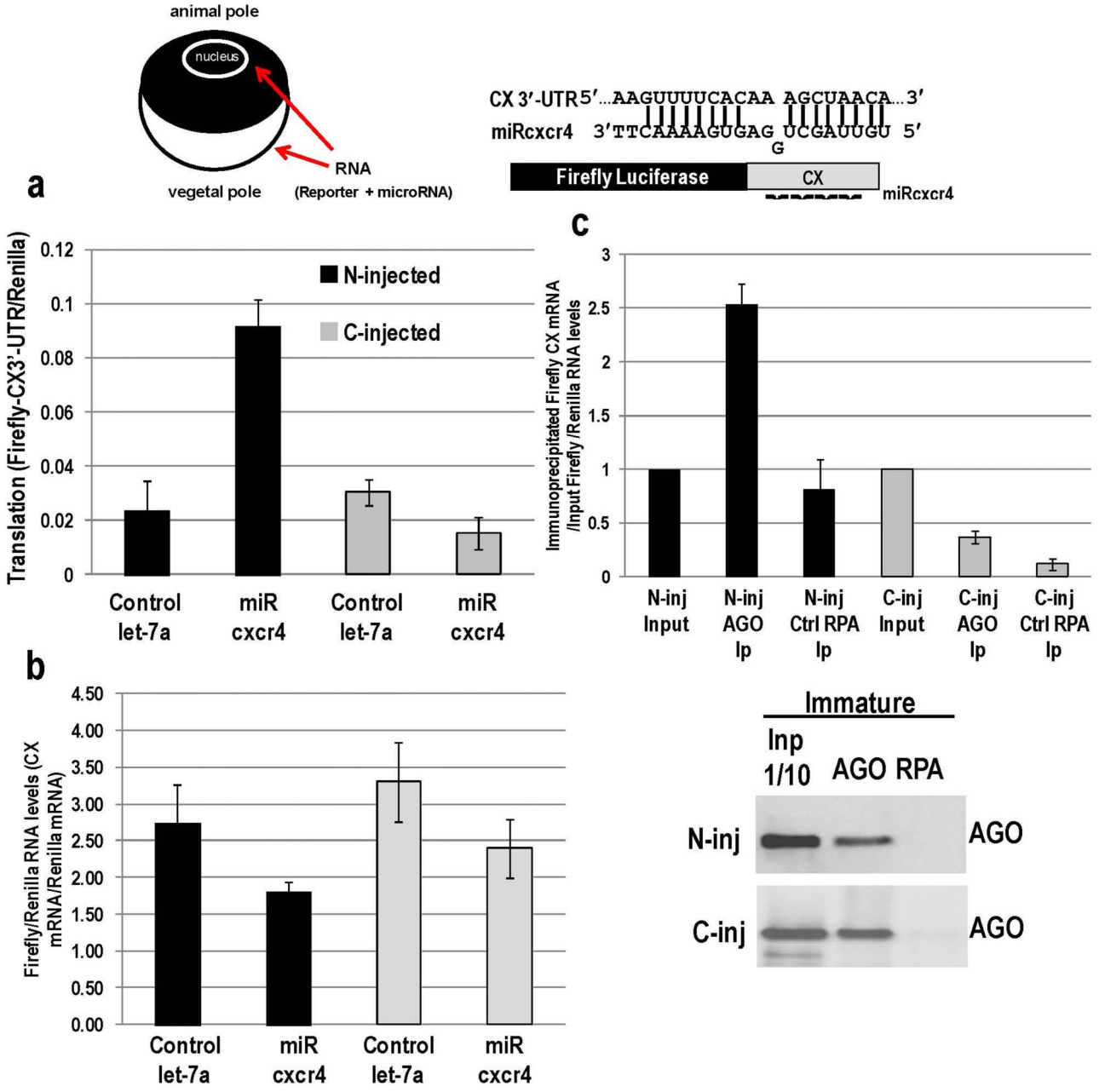

Figure 1 | Upregulated expression requires injection of the microRNA and target mRNA into the nucleus. (a) Nuclear injections (black bars) demonstrate upregulated expression of the CX reporter with miRcxcr4 and not with control let-7a while cytoplasmic injections demonstrate modest repression (gray bars). Titrating the reporter (nuclear injections at 0.3 fmols or 0.03 fmols of reporter mRNA, shown 0.03 fmols) maintains activation (injections performed with RNA as depicted above[38]); cytoplasmic injections (at 0.03 fmols of reporter mRNA (shown) or lower at 0.001 fmols-0.0001 fmols or higher upto 3.3 fmols) show no significant changes while increasing the injected microRNA levels does not stimulate[38]. MicroRNA half-lives and export were demonstrated previously[38] (b) mRNA levels of the CX reporter normalized to Renilla reporter levels as analyzed by qRT-PCR do not reflect the changes observed in the translation levels with nuclear or cytoplasmic injections. (c) AGO2 and control RPA antibody immunoprecipitates (Ips, Western blots shown below) from nuclear or cytoplasmically injected (N-inj or C-inj, injected with CX reporter, Renilla reporter and miRcxcr4) oocytes were analyzed by qRT-PCR for the target CX reporter and control Renilla reporters compared to 10% Input samples. Approximately 25% of CX reporter is detected associated with AGO2 in the nuclear injected oocytes compared to 4% in the cytoplasmically injected oocytes. The average values of three replicates with standard deviations as error bars are shown in a-c.

immunoprecipitations from isolated nuclei revealed endogenous Myt1 mRNA associated with AGO but not with control (RPA) antibody (Fig. 4a–b). Cyclin A1 mRNA, which is repressed in immature oocytes[32] and tRNAlys were not recruited by nuclear AGO and served as controls (Fig. 4a). These data indicate that the nuclear AGO complex recruits specific mRNAs that include targets for activation but not mRNAs targeted for repression or non-related RNAs.

FXR1 associates with AGO in the oocyte nucleus. Previous reports demonstrated a 160 kDa AGO2 complex in mammalian nuclei[41], indicating the absence of GW182 (~182 kDa) in this complex (AGO2, 90–100 kDa+GW182, 182 kDa) and suggesting the potential presence of FXR1-iso-a, (56–60 kDa), which is required for translation activation[38,53–56]. Immunoprecipitation of AGO from oocyte nuclei and cytoplasm revealed the association of *Xenopus*

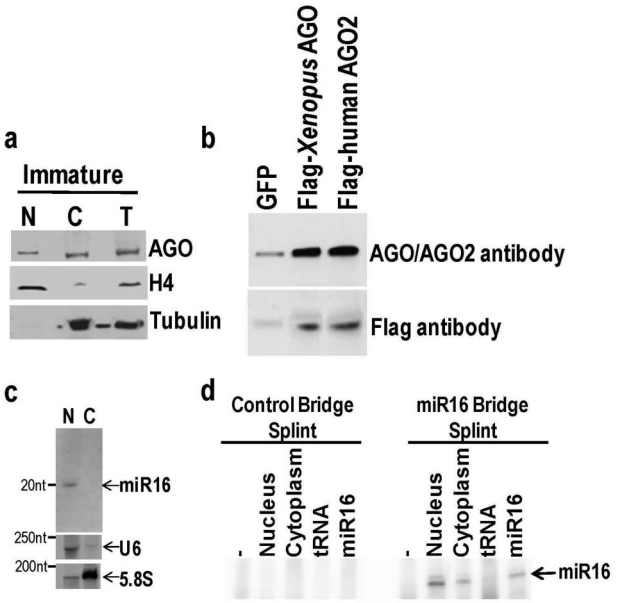

Figure 2 | *Xenopus* AGO and an endogenous microRNA, miR16, are substantially present in the nucleus in immature oocytes. All extracts (RNA and protein) and nucleus/cytoplasm isolation were prepared as described[37,38] (Methods). (a) Western analysis of total (T), nuclear (N) and cytoplasmic (C) sonicated extracts from 20 immature, folliculated oocytes using histone H4 as a marker for nuclei and tubulin as a cytoplasmic marker. A lane was left after each fraction loaded to preclude signals from spillover (observed with the tubulin signal in the lane between the cytoplasm and total samples). AGO is more clearly detected when cytoplasmic extracts are sonicated (shown) than in soluble cytoplasmic extracts (all other figures)[38]. (b) The antibody used for AGO analysis, detects expression from Flag-tagged *Xenopus* AGO and human (hAGO2) clones. *In vitro* transcription coupled translation RRL extracts (Promega) were used to express control GFP, Flag-tagged *Xenopus* AGO and human AGO2 (hAGO2) that were further subject to Flag purification and subsequent Western analyses with the AGO2 antibody used (Millipore, AGO2 antibody) and cross-checked with Flag antibody. (c) Northern analysis of endogenous miR16 in nuclei and cytoplasm of immature oocytes using probes against U6 as a nuclear marker and 5.8S rRNA as a cytoplasmic marker. Endogenous miR16 is substantially nuclear. (d) Splint ligation reactions for detection of miR16/B3 5′ trimmed form using a specific B3 or a control bridging oligonucleotide (bridge splint) as described previously[38] with nuclear and cytoplasmic oocyte RNA samples from 20 oocytes each. *In vitro* synthesized miR16/B3 form was used as a positive control and tRNA as a negative control.

FXR1, but in substantially greater amounts from the nuclei (Fig. 4b), indicating a nuclear AGO-FXR1 complex.

Overexpression of FXR1-iso-a leads to increased cytoplasmic AGO levels and rescues upregulated expression with cytoplasmically injected targets and microRNAs. The nuclear localization of microRNP components (Fig. 2) suggests a nuclear phase for the microRNP, also recently suggested for mammalian cells[1,8,41,50,57,60]. Notably, an AGO-FXR1 complex required for translation activation is more substantially present in the nucleus than in the cytoplasm (Fig. 4b). These data indicate that nuclear injections may be required to enable recruitment of the microRNA and subsequently of the mRNA to the predominantly nuclear AGO-FXR1 activation complex, leading to downstream activation of the target mRNA in the cytoplasm. To test this hypothesis, we asked whether increased expression of FXR1-iso-a could rescue activation when the reporter mRNA and microRNA are cytoplasmically injected. Fig. 5a shows that increased expression of FXR1-iso-a leads to rescue of activation with cytoplasmic injections of CX mRNA and miRcxcr4 but not with a control microRNA or upon expression of control GFP. The reporter RNA levels did not correlate with the translation outcome and could not account for the expression changes (Fig. 5c). Significantly, Western blot analyses of nuclear and cytoplasmically fractionated samples revealed an increased of AGO in the cytoplasm of FXR1-iso-a-expressing oocytes compared to the GFP control-expressing oocyte cytoplasm (Fig. 5b). We hypothesized that target activation resulted from increased levels of FXR1-iso-a and AGO in the cytoplasm, which would increase their interaction. Consistently, upon FXR1-iso-a expression, increased presence of AGO-FXR1 complex was observed in the cytoplasm (Fig. 5d), correlating with upregulated translation of the cytoplasmically-injected reporter and corresponding microRNA (Fig. 5a). In contrast, when control GFP was expressed (Fig. 5d), no increase in cytoplasmic AGO levels was observed and translation was not activated (Fig. 5a). These data suggest that the presence of AGO-FXR1-iso-a complex enables translation activation.

AGO2, FXR1 and microRNAs are present in human nuclei. To test whether these results were also true for mammalian G0 cells, we fractionated human THP1 cells grown in cycling or G0 conditions. The nuclei and cytoplasm were subjected to Western blot analyses, as well as qRT-PCR analyses for microRNAs. The purity of the fractions was ascertained by probing for CBP80 and tubulin as nuclear and cytoplasmic markers respectively. Fig. 6a shows Western blot analyses of nuclear-cytoplasmically fractionated THP1 cells. Although AGO2 levels is greater in the cytoplasm, we detect AGO2 in the nuclei of growing cells as previously observed[1,8,41,50,57,60] and in G0 cells. AGO proteins have been previously reported to be associated with CRM1, which is involved in nuclear export/import of specific classes of RNAs, including some microRNAs[8,60]. CRM1 transport is sensitive to Leptomycin B (LMB) inhibition[8], while FXR1 bears a nuclear localization signal (NLS) and a CRM1-dependent nuclear export signal (NES) and was demonstrated to be exported partially via CRM1[49]. We find that LMB treatment causes partial retention in nuclear levels of AGO2 and FXR1 but not of the controls, CBP80 or tubulin, indicating that the signal observed upon nuclear fractionation is presence in the nucleus and not contamination with cytoplasmic material (Fig. 6a). RT-PCR analysis demonstrated the presence of microRNAs in both nuclear and cytoplasmic compartments (Fig. 6b, miR16 shown), as has been observed for several microRNAs[8,23,30,44,57,60].

Nuclear AGO2 associates with an activated but not a repressed target in G0 human cell nuclei. To test whether nuclear AGO2 recruits mRNAs selectively in the nucleus, AGO2 was immunoprecipitated from G0 and non-G0 fractionated samples after *in vivo* formaldehyde crosslinking[40,54] to freeze and preserve endogenous complexes. AGO2 immunoprecipitation (Fig. 7c) revealed nuclear AGO2 associated with endogenous TNFα mRNA, a target for G0 activation, in the nucleus of G0 cells but not in growing cells, 4 hrs after initiating serum-starvation (Fig. 7a, first panel). Cyclin E mRNA, which is repressed in G0 cells, is not associated with AGO2 in the nucleus but in the cytoplasm (Fig. 7a, second panel), as expected for repressed RNAs[34,57]. After a longer (8 hrs) serum-starvation, TNFα mRNA is also found associated with AGO2 in the G0 cytoplasm (Fig. 7a, third panel). These data suggest that specific mRNAs may be recruited to AGO complexes in the nucleus, which enables them to be directed for activation in the cytoplasm. To test whether nuclear AGO recruitment was specific for the target sequence on TNFα mRNA, we tested Firefly reporters that bear 1) a wild-type TNFα mRNA 3′-UTR AU-rich element (ARE), which is activated in G0 cells and 2) a mutant sequence (mtARE), which fails to be activated[53–56]. As shown in Fig. 7b, the ARE reporter, which is activated in G0, associates considerably with nuclear AGO2 in G0, suggesting that the TNFα mRNA is recruited by AGO2 in the nucleus

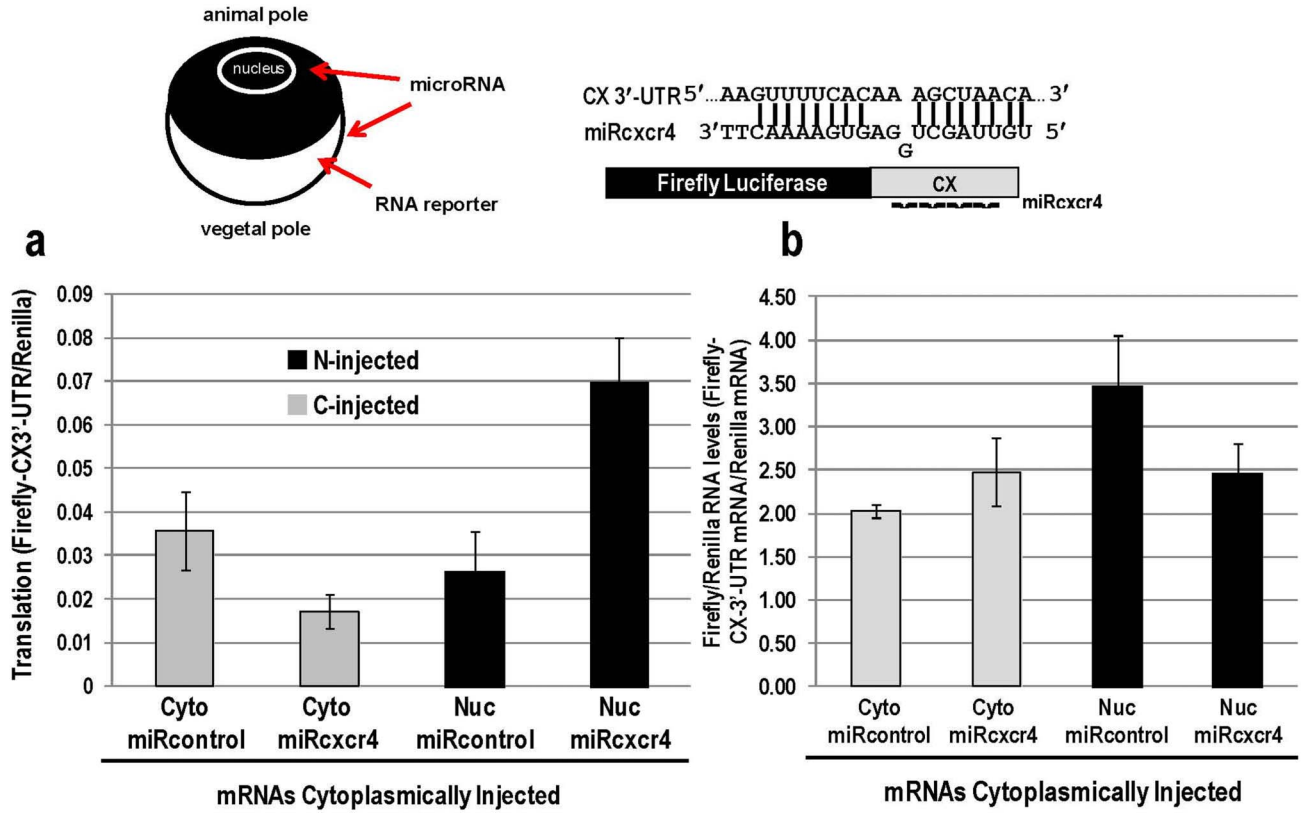

Figure 3 | Upregulated expression requires injection of the microRNA into the nucleus. (a) Nuclear injections (black bars) of miRcxcr4 but not control let-7a (miRcontrol) with cytoplasmic injection of the CX and Renilla reporters rescues upregulated expression of the CX reporter unlike cytoplasmic injections of miRcxcr4, where modest repression is observed (gray bars); injections were performed with transcribed RNA as depicted above. (b) mRNA levels of the CX reporter normalized to Renilla reporter levels as analyzed by qRT-PCR with nuclear or cytoplasmic injections of the microRNA do not correlate with translation changes. The average values of three replicates with standard deviations as error bars are shown in a-b.

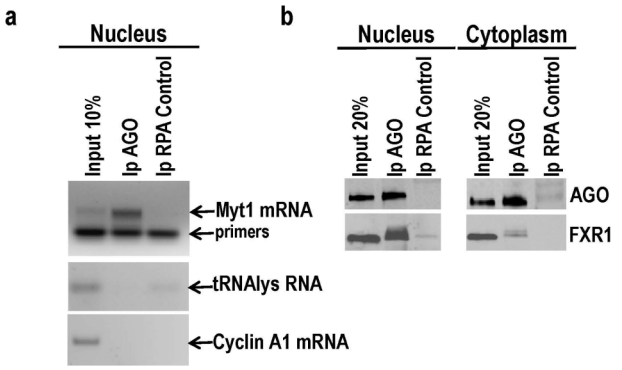

Figure 4 | Myt1, an endogenous target mRNA for activation by miR16, associates with AGO in the nucleus where AGO interacts substantially with FXR1, a co-factor required for activation. (a) Immunoprecipitation of *Xenopus* AGO (anti-AGO/2A8 antibody[39]) or control RPA from nuclear extracts followed by RT-PCR analysis demonstrated that Myt1 mRNA was specifically associated with AGO in the nucleus; Cyclin A1 mRNA, which is repressed in immature oocytes[32] and tRNAlys served as negative controls to test specificity. (b) Immunoprecipitation of AGO (anti-AGO/2A8 antibody[39]) or control (RPA antibody) from nuclear and cytoplasmic extracts followed by Western blot analyses using anti-AGO2 (Millipore) and anti-FXR1 (Abcam). AGO immunoprecipitates demonstrated association with FXR1 in both the nucleus and cytoplasm, with more substantial co-immunoprecipitation of FXR1 with AGO in the nucleus, indicating the presence of a prominent nuclear AGO-FXR1 complex.

via the target ARE (Fig. 7b) in G0[55]. Therefore, specific mRNA targets for activation, such as Myt1 (Fig. 4a) and TNFα (Fig. 7a–b), are selectively recruited by a nuclear RNP containing AGO2; mRNAs targeted for repression, like Cyclin A1 (Fig. 4a) and Cyclin E (Fig. 7a), are not.

FXR1-iso-a interacts with AGO2 in human nuclei. An mRNA associated with an AGO2 complex that lacks GW182 is known to derepress or activate mRNA expression[26,51] while FXR1-iso-a tethering to an mRNA also leads to its activated translation[54]. A previously observed nuclear AGO2 complex of 160 kDa is too small to contain GW182 (182 kDa)[41]; the size of this complex is consistent with AGO2 (90–100 kDa) plus FXR1-iso-a (56–60 kDa), similar to the nuclear complex in oocytes (Fig. 4b). *In vivo* formaldehyde crosslinking[40,54] followed by nuclear-cytoplasmic fractionation and then immunoprecipitation of AGO2 from the nuclear and cytoplasmic fractions revealed that AGO2 interacts substantially with FXR1 in the nucleus (cytoplasmic interaction can be observed upon extended exposure but is reduced, data not shown) of cycling and G0 cells (Fig. 7c). In the cytoplasmic fraction of G0 cells, the antibody immunoprecipitated less AGO, possibly due to the epitope being masked. It remains possible that some amount of the complex, not detected by the antibody, is also present in the G0 cytoplasm, which is consistent with its role in translation activation in G0. However, in the cytoplasmic fraction of cycling cells, AGO is immunoprecipitated at equal levels to that in the nucleus but FXR1 association is clearly reduced compared to the nucleus, indicating that the complex is primarily nuclear in such cells (Fig. 7c). Since TNFα and its regulatory microRNA are induced in G0 and reduced in asynchronous cycling cells[53–56], the AGO-FXR1 complex does not recruit the TNFα mRNA in cycling cells (Fig. 7a). Cytoplasmic AGO2 is associated

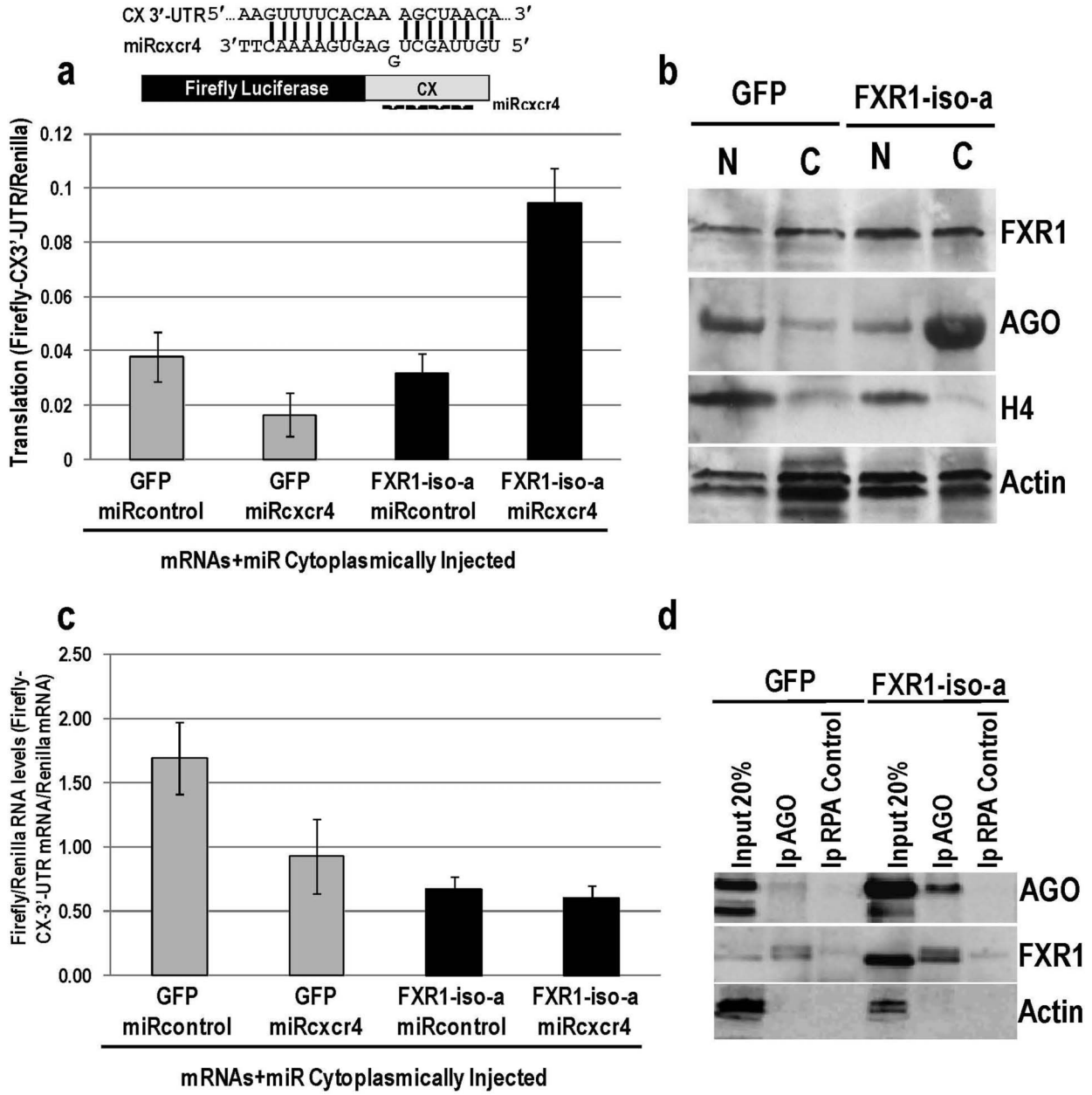

Figure 5 | Expression of FXR1-iso-a to overexpress FXR1 levels increases the cytoplasmic levels of AGO and rescues translation activation of CX mRNA by miRcxcr4, injected cytoplasmically into oocytes. (a) Oocytes were nuclear injected with DNA plasmids to express either GFP control or FXR1-iso-a in oocytes and were subsequently cytoplasmically injected with miRcxcr4 or control let-7a, CX and Renilla reporters. Upregulated expression of the CX reporter in the presence of miRcxcr4 was rescued upon overexpression of FXR1-iso-a but not with GFP control or with the control microRNA. (b) Western blot analysis of nuclei (N) and cytoplasm (C) extracts (soluble extracts that were not sonicated) from 20 immature, folliculated oocytes each that were injected with DNA plasmids to express either GFP control or FXR1-iso-a. Histone H4 and actin served as controls. FXR1-iso-a expression leads to increased FXR1 levels as well as increased cytoplasmic levels of AGO. AGO is more clearly detected when cytoplasmic extracts are sonicated (Fig. 2a) compared to soluble cytoplasmic extracts (this figure)[38]. (c) mRNA levels of the CX reporter normalized to Renilla reporter levels as analyzed by qRT-PCR with GFP or FXR1-iso-a expression do not correlate with translation changes in (a). (d) AGO2 (Wako) immunoprecipitates from cytoplasmic samples expressing either GFP control or FXR1-iso-a were analyzed for co-immunoprecipitation of FXR1. Increased levels of FXR1 were immunoprecipitated with samples overexpressing FXR1-iso-a. RPA antibody immunoprecipitation served as an antibody control. Increased AGO and FXR1 levels can be observed in the Input lane of the samples overexpressing FXR1-iso-a compared to Actin levels used as a loading control; the AGO antibody amounts (1/3) used for immunoprecipitation are limiting. The average values of three replicates with standard deviations as error bars are shown in a, c.

with larger complexes (upto 2–3 MDa[41]) in particular, with the negative regulator, GW182[4,10,13,16,33,58], and microRNAs[57] and are repressive. Immunoprecipitation with antibodies against the GW182/P body (anti-18033[58,59]) to co-precipitate RNAs with repressive GW182 complexes did not detect TNFα mRNA in the nucleus or cytoplasm (Supplementary Fig. S1, first panel). Cyclin E mRNA, which is repressed in G0[34], was detected associated with GW182 complexes, in particular, in the cytoplasmic but not in the nuclear complex in G0 cells. These data indicate that repressed mRNAs like Cyclin E, but not activated mRNAs like TNFα are recruited to cytoplasmic GW182-AGO2 repressive complexes (Supplementary Fig. S1, second panel and Fig. 7a). A similar experiment could not be performed with anti-FXR1-iso-a[53–56], due to the lack of isoform-specific antibodies. Compartmentalization may enable selective recruitment of specific

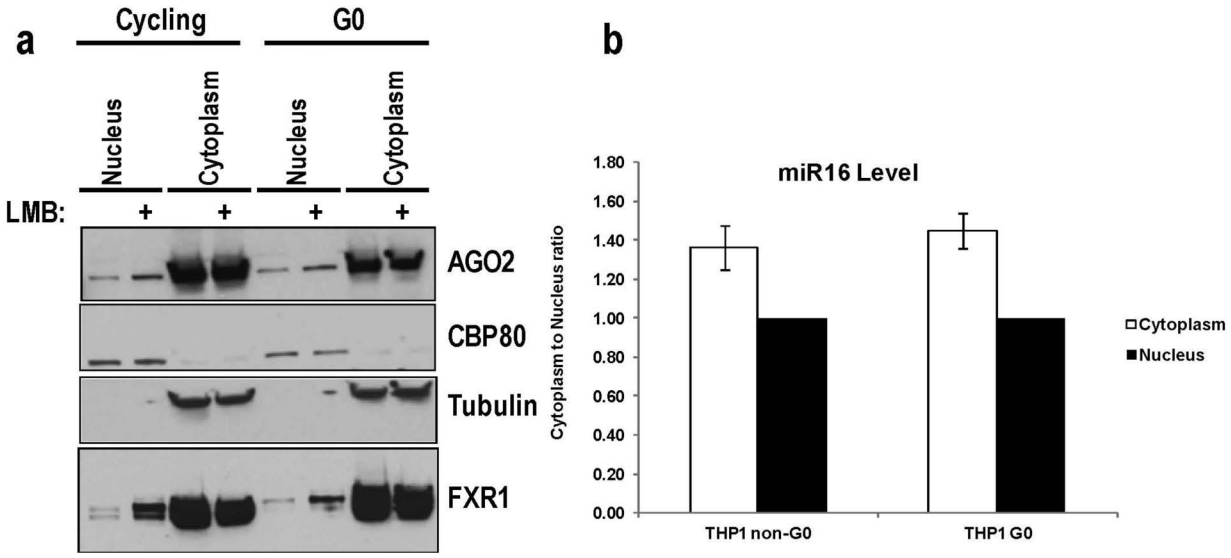

Figure 6 | AGO2, FXR1 and microRNAs like miR16 are present in mammalian nuclei and are partially restricted by LMB inhibition of the CRM1 export pathway. (a) Western analysis of AGO2 and FXR1 in nuclear and cytoplasmic extracts from proliferating (Cycling) and G0 THP1 cells that were untreated or treated (+)with LMB to inhibit the CRM1 pathway[8,49]. CBP80 served as a marker for nuclei and tubulin as a cytoplasmic marker. (b) MicroRNAs are present in the nucleus and cytoplasm as previously published[8,23,30,44,57,60]. MiR16 levels in proliferating (non-G0) and G0 cell nuclei and cytoplasm as analyzed by qRT-PCR. The average values of three replicates with standard deviations as error bars are shown.

microRNAs and their targets into nuclear AGO2-FXR1-iso-a complexes, previously demonstrated to be required for activation. These RNAs may be specifically induced or mobilized in G0 and therefore, are recruited in the nuclear compartment while pre-existing, proliferation-associated mRNAs are cytoplasmic and not re-cruited by the nuclear activation complex (TNFα mRNA is recruited by the nuclear activation complex but Cyclin E mRNA is not, Fig. 7a–b)[34,53–56].

Overexpression of FXR1-iso-a leads to increased cytoplasmic AGO2 levels and rescues upregulation of expression by microRNAs in asynchronous, low-density proliferating cells. We had earlier observed that overexpression of FXR1-iso-a in low density proliferating cells promoted translation[54], similar to the rescue of activation of cytoplasmically-injected reporters and microRNA after FXR1-iso-a expression in oocytes (Fig. 5a). Significantly, nuclear-cytoplasmic fractionation of low density proliferating cells (Fig. 8a, histone and tubulin serve as markers for the nucleus and cytoplasm, respectively), overexpressing FXR1-iso-a (Fig. 8a, FXR1) but not control GFP, demonstrated increased presence of AGO2 in the cytoplasm (Fig. 8a, AGO2, short exposure) with some increase also detected in the nucleus. Although more AGO2 and FXR1 are present in the cytoplasm than in the nucleus of mammalian cycling cells (Fig. 6a), an AGO2-FXR1-iso-a complex was not significantly detected in the cytoplasm (Fig. 8b, GFP and Fig. 7c) of asynchronous, low-density proliferating cells (slowly proliferating, mostly lag phase cells) and is reduced in oocytes (Fig. 4b). Upon increased expression of FXR1-iso-a in asynchronous, low density proliferating cells, an AGO2-FXR1-iso-a complex (Fig. 8b, FXR1 lanes, anti-FXR1) could be immunoprecipitated from cytoplasmic extracts. Consistently, increased expression of FXR1-iso-a led to activation of translation of CX reporter mRNA mediated by miRcxcr4 but not by a control microRNA (Fig. 8c) in these asynchronous, low-density proliferating cells. In high density proliferating cells, repression is dominantly observed with microRNAs[24,53,55,56] and overexpression of FXR1-iso-a non-specifically increased general translation (data not shown). These results suggest that the increased levels of the AGO2-FXR1-iso-a complex facilitate activation. This occurs naturally in nuclei with nuclear recruitment of specific mRNAs in oocyte nuclei and in mammalian G0 cells (Figs. 1–4, 6–7) but can be induced in the cytoplasm in oocytes and in low-density proliferating mammalian cells through overexpression of FXR1-iso-a (Figs. 5, 8).

Discussion

Translational activation of specific mRNAs in alternative cell states, such as the immature oocyte and mammalian G0 cells, provides a means to maintain that state for extended durations[45]. The immature oocyte post-transcriptionally regulates the expression of genes essential for maintaining the immature state and for subsequent oocyte maturation[19]. MicroRNPs can activate translation of specific reporters in G0 mammalian cells[31,52–54]. MicroRNP-controlled activation, mediated by AGO and miR16, is required in part to maintain the immature state in the naturally quiescent-like *Xenopus laevis* immature oocyte[38]. Here, we have found that activation involves selective microRNA and target recruitment by a distinct, nuclear AGO-FXR1 complex in immature oocytes and G0 human cells, coincident with the requirement for a compartmentalized, nuclear phase to enable microRNA-mediated upregulation of specific reporter mRNAs in immature *Xenopus laevis* oocytes.

A primary feature common to the two systems—quiescent cells and immature oocytes—is the presence of an intact nucleus. We find that microRNAs and their target mRNAs injected into the oocyte nucleus are recruited for translation activation by microRNPs (Figs. 1,3), while those injected into the cytoplasm or into mature oocytes (lacking nuclei) lead to repression, consistent with the absence of a nuclear phase or of an intact nucleus after Germinal vesicle breakdown (GVBD)[38] or during the cell cycle[56]. The endogenous microRNA, miR16, is detected in the nucleus (Fig. 2C–D), as are AGO and FXR1 (Figs. 2a–4b), which are required for activation of a target mRNA[38]. MicroRNAs such as miR206, implicated in specific activation in quiescent-like conditions[31], have also been observed in mammalian cell nuclei[44]. While worms express an Argonaute with a specialized NLS[21], human AGO2 along with associated microRNAs can be imported into the nucleus[8,41,50,57,60] via Importin 8 and associations that lead to CRM1-mediated transport[8,57,60]. Importin 8 antisense in the oocyte affected viability and could not be tested (data not shown). AGO2 and FXR1 demonstrated partial nuclear retention (Fig. 6a) upon LMB blockage of CRM1, similar to the effects previously observed with microRNAs and FXR1 with LMB

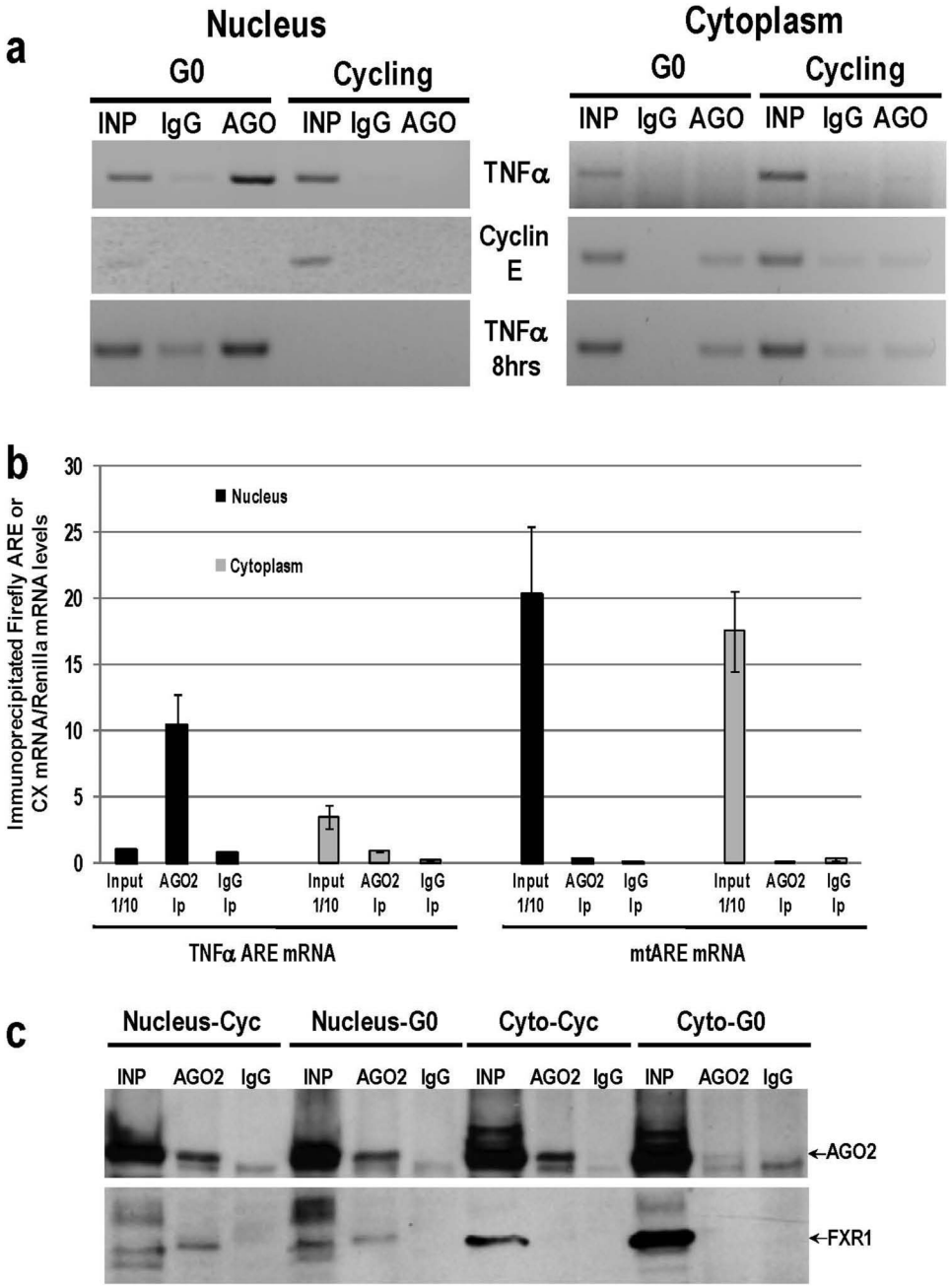

Figure 7 | AGO2 interacts substantially with FXR1 and associates with activated but not repressed targets in G0 human cell nuclei. All interactions were observed after *in vivo* formaldehyde crosslinking[40,54] followed by nuclear-cytoplasmic fractionation and immunoprecipitation. (a) Immunoprecipitation of AGO2 (Wako) or control IgG from nuclear and cytoplasmic extracts of proliferating (Cycling) and G0 THP1 cells followed by RT-PCR analysis demonstrated that TNFα mRNA was specifically associated with AGO2 exclusively in G0 nuclei after 4 hrs of serum-starvation induction of G0 (TNFα, first panel, 4 hrs G0) and also in the G0 cytoplasm later at 8 hrs of serum-starvation (TNFα, third panel, 8 hrs G0). Cyclin E mRNA, which is repressed in G0[34], served as a control and is not present in the nuclear complex but in the G0 cytoplasmic complex (Cyclin E, second panel, 4 hrs G0; the mRNA is downregulated and below detection by 8 hrs). Input (INP) = 10% of the sample. (b) G0 HEK293 cells were transfected with reporters that bear the AU-rich target sequence of TNFα mRNA (TNFα ARE, sufficient for activation in G0[54,55]) or a mutated sequence (mtARE, non-functional for activation)[54,55]. Immunoprecipitation of AGO2 or control IgG from nuclear and cytoplasmic extracts from transfected late G0 cells followed by RT-PCR analysis demonstrated that the ARE reporter was associated substantially with AGO2 in G0 nuclei, with some association in G0 cytoplasm as expected at late G0, similar to (a). No association was observed with the mutated (mtARE) reporter; tRNAlys and Renilla served as controls. The average values of three replicates with standard deviations as error bars are shown. (c) Western analyses of immunoprecipitates from 4hr G0 and cycling (Cyc) samples from (a) using anti-AGO2 (Millipore) and anti-FXR1. Input (INP) =10% of the sample. A non-specific band immunoprecipitated with anti-AGO2 and IgG in the AGO2 Western blot analysis below the specific AGO2 band. Anti-FXR1 recognizes several bands in the Input lane representing seven isoforms and modifications. In G0 cytoplasmic fractions (Cyto-G0), the antibody immunoprecipitated less AGO, possibly due to the epitope being masked. In cycling cell cytoplasmic fractions (Cyto-Cyc), AGO is immunoprecipitated at equal levels to that in the nucleus but FXR1association is clearly reduced. AGO2-FXR1 complex is substantially present in the nucleus.

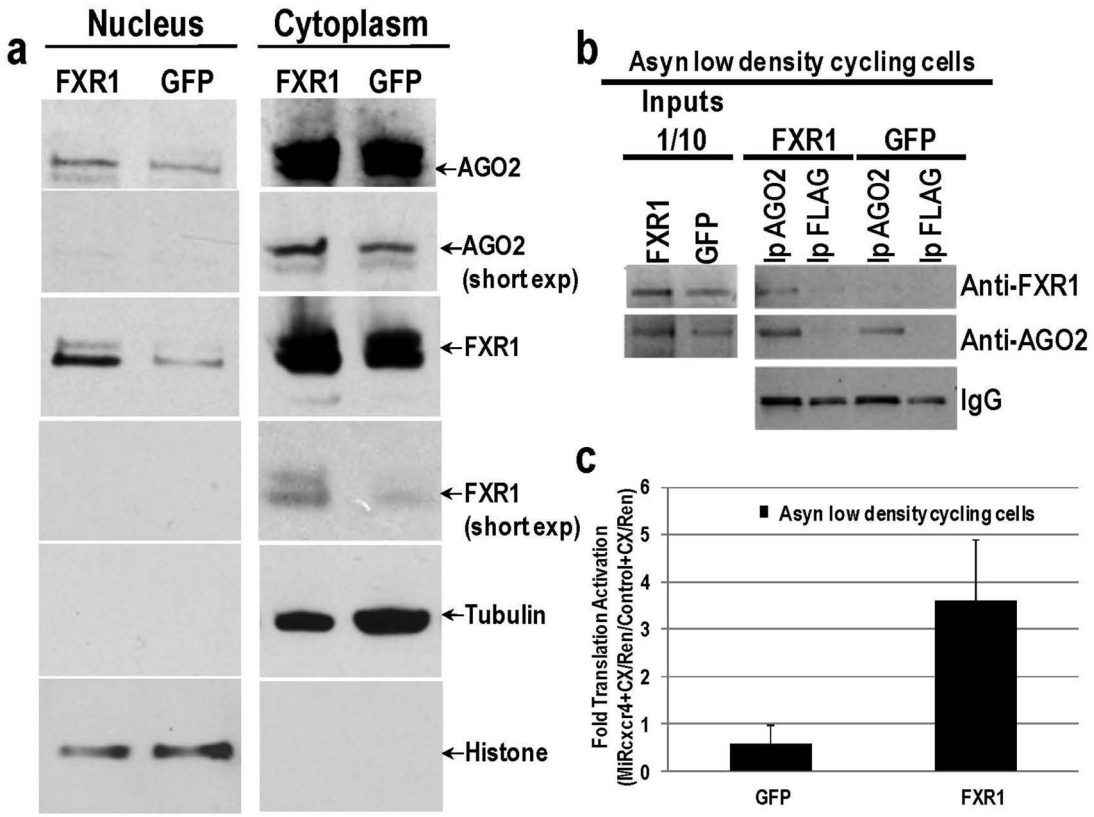

Figure 8 | Expression of FXR1-iso-a to overexpress FXR1 levels increases the cytoplasmic levels of AGO2 and rescues translation activation of CX mRNA by miRcxcr4 in asynchronous, low density proliferating cells. (a) Western analysis of nuclear and cytoplasmic extracts from asynchronous, low density proliferating HEK293 cells (slowly proliferating, mostly lag phase cells) that express either GFP control or FXR1-iso-a (FXR1, 1μg transfected/ $1x10^5$ cells/ml). FXR1-iso-a overexpression leads to increased FXR1 levels as well as increased cytoplasmic AGO2 levels with some increase in the nucleus (short exp=short exposure of the saturated levels present in the original exposure above). Histone H4 and tubulin served as nuclear and cytoplasmic markers, respectively. (b) Immunoprecipitation of AGO2 from cytoplasmic extracts expressing FXR1-iso-a or GFP in asynchronous, low density proliferating cells demonstrated increased interaction between AGO2 and FXR1-iso-a upon FXR1-iso-a but not GFP expression. (c) Expression of either GFP control or FXR1-iso-a in asynchronous, low density proliferating cells that were subsequently transfected with miRcxcr4 or let-7a (control), CX and Renilla (Ren) reporters, demonstrated rescued upregulated expression of the CX reporter in the presence of miRcxcr4 upon overexpression of FXR1-iso-a but not GFP control. The fold activation depicted (fold activation = the Firefly Luciferase translation value of CX reporter observed in the presence of miRcxcr4 normalized to Renilla over the Luciferase value of CX with control Let-7a microRNA normalized to Renilla that were further normalized to their RNA levels, which do not change significantly as described previously)[54] are compared between samples overexpressing GFP or FXR1-iso-a. The average values of three replicates with standard deviations as error bars are shown.

treatment[8,49,57,60]. The partial retention observed is likely due to only partial inhibition of CRM1 export by LMB[8,49] and not complete loss of CRM1 functions upon CRM1 depletion[60]. The AGO-FXR1 complex was previously detected in multiple AGO complex purifications[9,25]. While tethering AGO2 or GW182[4,54] to a reporter in cycling cells promotes repression, tethering FXR1 does not[54]. In quiescent conditions, FXR1 and AGO2 promoted expression of the tethered reporter[54], dependent on each other, indicating that the complex was required for activation. These studies suggest a compartmentalized role for a specific AGO2-FXR1-iso-a complex in the nucleus that leads to translation activation of recruited mRNAs in the cytoplasm in G0 and immature oocytes.

Although more AGO2 is present in the cytoplasm than in the nucleus of mammalian cells (Fig. 6) and the target mRNA is stable in oocytes in the nucleus or cytoplasm[38] (Fig. 1b), cytoplasmic AGO2 is bound in larger complexes (upto 2–3 MDa[41]) in particular, with the negative regulator, GW182[4,10,13,16,33,58], and microRNA[57], and is repressive. GW182 can be detected in mammalian nuclei; however, the nuclear AGO interacting complex detected is smaller (160 kDa[41]) and therefore lacks the repressive factor GW182. AGO2-GW182 interaction is altered in G0[58] and mouse oocytes[18,36]. Immunoprecipitation with anti-18033 to detect P bodies/GW182[58,59] in oocytes, co-immunoprecipitated an AGO detected with an antibody against the PIWI domain (but not with the AGO2 antibody used to detect our clone as previously published[35]) in the cytoplasm, suggesting that AGO-GW182 interaction was detected with potentially a distinct AGO in the cytoplasm. Tagged proteins could not be used for better detection of GW182 only (and not GW182/P body complexes[58,59]) as expression of tagged GW182 has a dominant repressive effect, which precludes its use to study endogenous complexes during activation (data not shown). Anti-18033 does not co-precipitate FXR1 in oocytes and mammalian cells while GW182 was not detected in FXR1 immunoprecipitates (data not shown). These data indicate separate AGO2 interactions and complexes with the repressive co-factor, GW182, and with the activating co-factor, FXR1-iso-a, leading to distinct translation outcomes of associated transcripts.

Endogenous AGO2-FXR1-iso-a complex was not detected in the cytoplasm (Fig. 8b, GFP and Fig. 7c) of asynchronous, low density proliferating cells and was reduced in oocyte cytoplasm (Fig. 4b). Overexpression of FXR1-iso-a led to increased association with AGO2 in the cytoplasm of oocytes and low density slowly proliferating cells (Figs. 5d, 8b), substituting for the nuclear AGO complex

and inducing activation (Figs. 5a, 8c). How AGO2 levels increase in the cytoplasm upon FXR1-iso-a overexpression (increased AGO2 export, translation and/or protein stability mediated by FXR1-iso-a, Figs. 5b, 8a) remains to be investigated. Increased AGO2-FXR1-iso-a complexes (Figs. 5d, 8b), provide the cytoplasm with activation complexes to enable recruitment of the microRNA and mRNA and consequent activation (Figs. 5a, 8c). These data indicate that increased presence of the activation complex, naturally compartmentalized in the nucleus or upon exogenous FXR1-iso-a overexpression, contributes to selective recruitment for activation of distinct mRNAs by microRNAs (Figs. 4, 5, 7, 8).

Many mRNAs in the oocyte and in mammalian cells associate with specific RNA-binding proteins in the nucleus that then dictate appropriate fates in the cytoplasm[20,29,32]. microRNAs[8,23,30,44,57,60], microRNPs[1,8,41,50,57,60] and RNA binding proteins[1,2,8,14,15,28,41,49,50,57,60], in particular, FXR1[2,14,15,28,49], have been demonstrated to associate with target mRNAs in the nucleus. Specific microRNAs in G0, compartmentalized in the nucleus, may selectively associate with the nuclear AGO2-FXR1-iso-a complex, to recruit target mRNAs, such as Myt1 and TNFα mRNAs, for upregulated translation (Figs. 2c–d, 4, 6–7). These RNAs are induced or mobilized in G0 for conducting G0-related functions and may thus be available in the nucleus for recruitment. Cytoplasmic microRNAs (induced in G0 or pre-existing) are recruited into repressive cytoplasmic AGO complexes[57] that lack AGO2-FXR1-iso-a (Figs. 4b, 7c, cytoplasm FXR1 blot), thereby, directing them towards downregulation of pre-existing target mRNAs associated with proliferation, such as Cyclin E mRNA, which was translated during the cell cycle prior to arrest[11,34] and would be cytoplasmically present (Fig. 7a and Supplementary Fig. S1, Cyclin E-recruitment by AGO2 and GW182 complex in G0 cytoplasm). Regulated expression and modifications of the microRNP, microRNA or mRNA in G0 may also contribute to enable specific recruitment and activation[53–56,26,38]. Our data suggest that in immature oocytes and human G0 cells, the nuclear phase of microRNAs and AGO2-FXR1-iso-a complex (Figs. 2–4, 6–7) is consistent with selective recruitment to the nuclear AGO2-FXR1-iso-a complex, which may direct specific targets to undergo translation upregulation by microRNAs (Figs. 1, 3, 4, 7)[38,54,55].

Methods

Oocytes. Oocytes (Immature, folliculated stages IV-V oocytes) were harvested from human chorionic gonadotropin (hcG)-stimulated frogs by partial ovariectomy[37] and manipulated as described previously[37–38]. The pre-stimulated frogs were obtained from NASCO (WI). The oocyte removal and animal handling protocol[37] adhered strictly to the institutionally approved guidelines and regulations. The animal handling and oocyte removal protocols were reviewed, approved and renewed annually by the Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee (IACUC) for MGH.

Injections were performed using Drummond Nanojet II system with volumes of 18nls for reporters and plasmids (into the nucleus or cytoplasm). 18 nls containing 0.0625–0.125 ngs DNA plasmid, 0.3 or 0.03 fmols *in vitro*-transcribed Luciferase reporter mRNAs, 375 fmols microRNA were injected per oocyte with diluted blue dextran dye to visualize accurate delivery into the GV prior to harvest of the oocytes. For overexpression, 2.5 ngs of DNA plasmid (or 7 ngs of *in vitro*-transcribed, capped mRNA) of FXR1-iso-a or GFP was used. Translation was assayed with a time course ranging from 1–6 h and compared at 4 h in hcG-treated oocytes. All injections were conducted with folliculated immature stage IV-V oocytes. The injections were performed carefully to avoid escape of RNAs into the cytoplasm that would produce erroneously high background readings. All extracts (RNA and protein) and nucleus/cytoplasm isolation were prepared as described below[37,38] to prevent contamination of the samples with follicular material.

Plasmids and reporters. All plasmids, reporters and microRNAs used are described in[38]. Figs. 1,3 and 5 utilized *in vitro*-transcribed luciferase reporter RNAs. Unadenylated reporters were used as polyadenylated reporters precluded analysis of stimulated expression[38].

Luciferase assay. Extract preparation and luciferase assays were performed as described in the manufacturer's protocol (Promega). Oocytes were manually crushed in PLB buffer (Promega) and clarified by centrifugation at 2000 rpm for 5 minutes. The average ratios of luciferase values from at least three replicates are depicted with the standard deviations as error bars. RNA levels were assessed in each experiment separately (not used to normalize the values graphed), performed at least three times.

Protein and RNA analyses. Total oocyte extracts, immunoprecipitations and RNA analyses are described[37,38]. Folliculated oocytes were injected and incubated prior to harvest. All extracts (RNA and protein) and nucleus/cytoplasm isolation were prepared after collagenase treatment of the harvested oocytes followed by manual defolliculation and examination to ensure complete defolliculation of oocytes[37,38] to prevent contamination of the samples with follicular material. For all gels, equal numbers (20) defolliculated oocytes upon harvest, isolated as described in[37,38] were run in each lane.

Extract preparation and oocyte immunoprecipitations. Soluble extracts[38] for immunoprecipitations were prepared using 20 oocytes per sample of total oocytes, isolated nuclei and cytoplasm that were prepared as extracts after manual defolliculation as well as collagenase treatment of the oocytes and examination to ensure complete defolliculation prior to extract preparation (as described in[37,38]) to prevent contamination of the samples with follicular material. The oocytes, nuclei and cytoplasm were manually crushed in 150 µl of lysis buffer X (100 mM NaCl, 40 mM Hepes 7.8, 6 mM $MgCl_2$, 0.05% NP40) and adding DTT and PMSF to 1 mM each. The extract was incubated on ice for 5 minutes. The oocytes were crushed, NaCl added to 400 mM for 2 minutes to get high salt conditions to extract nuclear material, then crushed followed by correction buffer (lysis buffer X without salt) to bring back the salt concentration of the extract to 100 mM. The extract was then incubated on ice for 30 minutes. Low amounts of AGO are present in extracts if the extracts are clarified at 10000 g. Therefore, a low-speed centrifugation (2000 g) was used to clarify the extracts for immunoprecipitations. A pipette tip was used to clear the lipid layer and the clarified extract removed to a new tube. The immunoprecipitations were carried out with these soluble lysates of 40–100 oocytes using anti-pan-AGO/2A8 antibody (Santa Cruz)[39] while all Western analyses were with anti-AGO2 antibody (Millipore) with soluble extracts of 20 oocytes (Figs. 1, 4–5).

To analyze AGO levels by Western blotting, in particular, in the cytoplasm where AGO is present in large complexes (2–3 MDa;[41], total oocytes (defolliculated upon harvest) were prepared as sonicated lysates only for Fig. 2a. Total defolliculated oocytes, nuclei and cytoplasm were lysed and extracted on ice in lysis buffer X as described above for soluble extracts, followed by clarifying the sample at 2000 g for 5 minutes to separate out and remove the lipid/polysaccharide layer. The rest (lysate and resuspended pellet) were mixed, made up to 0.3% SDS, extracted on ice, sonicated for 10 s with 30 s cooling 3 times, and the subsequent sonicated lysate clarified by centrifugation at 2000 g for 5 minutes to remove debris, followed by acetone precipitation of the sonicated samples[38].

The acetone precipitates of both soluble and sonicated extracts were resuspended in SDS loading buffer and fractionated by 4–20% gradient SDS-PAGE followed by Western blotting and further analyses. *Xenopus* samples were probed with anti-*Xenopus* AGO/AGO2 (anti-AGO2, Millipore), anti-FXR1 (Abcam), anti-Histone H4 (Cell Signaling), anti-Tubulin (Santa Cruz), and anti-Actin (Sigma).

For immunoprecipitations[38], one half of the soluble extract was diluted 2-fold with lysis buffer X and incubated with 20 µl of anti-AGO/2A8 antibody or anti-RPA as control antibody overnight with nutation at 4°C. The samples were then transferred to new tubes, 40 µl of protein G agarose (pre-blocked for 1 hr with 1mg/ml of tRNA and glycogen) added, followed by incubation with nutation at 4°C for 1 hr after which the beads were concentrated by centrifugation at 6000 g for 10 minutes. The agarose beads were washed with lysis buffer X without DTT and glycerol, with 0.1% NP40 four times, once with RIPA buffer (1% NP-40, 0.5% Na deoxycholate, 150 mM NaCl, 50 mM Tris pH 7.5, 0.05% SDS) and then transferred to a new tube for acetone precipitation followed by or directly resuspended in 6x loading buffer with 0.4 M DTT. For immunoprecipitations for RT-PCR assays (Figs. 1, 3, 4A), 0.5 µl of RNasin was added to the extract before incubation with the antibody and the immunoprecipitates were used for RNA extraction/analyses. 40–100 oocytes were used for each sample.

RT-PCR analyses. cDNA synthesis was performed using Random Primers (Invitrogen) and the cDNA was subjected to PCR amplification with 52°C annealing and 28–35 cycles for different cDNA preparation yields. For mammalian samples, primers to endogenous mRNAs, TNFα (TNF360-5: CCCAGGGACCTCTCTCTAATCA and TNF470-3: AGCTGCCCCTCAGCTTGAG) and as a control, to tRNAlys (trnalys-5: GCCCGGATAGCTCAGTCGGTAGAG and trnalys-3: CGCCCGAACAGGGACTTGAACCC), exogenously introduced Firefly, FF-F3 (TTCCATCTTCCAGGGATACG) and FF-R3 (ATCCAGATCCACAACCTTCG) and, as a control, Renilla, Ren1 (CCATGATAATGTTGGACGAC) and Ren2 (GGCACCTTCAACAATAGCATTG) were used. For oocytes, endogenous mRNA Myt1 was analysed using Myt3245-5 (CAGTATTGTTGAATATATCATGTAACC) and Myt3245-3 (CTGCCATTATCAAGCAGGAGCACTGC) and Cyclin A1 primers as described previously[32]. The endogenous control used was *Xenopus laevis* tRNAlys, (tRNAlys-5: CCCGCATAGCTCAGTCGGTAGAGC and tRNAlys-3: CCCGAACAGGGACTTGAACCC).

Quantitative RT-PCR. Random hexamer and FF3END3 were used for cDNA synthesis. FF3END3 (caatttggactttccgcccttcttggc) and FF10-5 (ggattacgtggccagtcaagtaacaaccg) were used to amplify CX Firefly reporter mRNA at

the 3′ end of the Firefly coding region. Human miR16 levels were detected using the TaqMan-microRNA Reverse Transcription kit and TaqMan-microRNA Assay (Applied Biosystems) according to the manufacturer's instruction.

Mammalian cell fractionation, immunoprecipitation, crosslinking procedures. These procedures were conducted as described previously[46,54,55] and is detailed in the Supplementary Information.

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Author contributions

SST and RDM conducted the majority of the experiments. MS, JCS, JHL and OLT conducted part of the experiments for figures 6 and 8. SV planned the experiments and prepared the manuscript. All authors reviewed the manuscript.

Additional information

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