eIF1A augments Ago2-mediated Dicer-independent miRNA biogenesis and RNA interference

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MicroRNA (miRNA) biogenesis and miRNA-guided RNA interference (RNAi) are essential for gene expression in eukaryotes. Here we report that translation initiation factor eIF1A directly interacts with Ago2 and promotes Ago2 activities in RNAi and miR-451 biogenesis. Biochemical and NMR analyses demonstrate that eIF1A binds to the MID domain of Ago2 and this interaction does not impair translation initiation. Alanine mutation of the Ago2-facing Lys56 in eIF1A impairs RNAi activities in human cells and zebrafish. The eIF1A–Ago2 assembly facilitates Dicer-independent biogenesis of miR-451, which mediates erythrocyte maturation. Human eIF1A (heIF1A), but not heIF1A(K56A), rescues the erythrocyte maturation delay in eif1axb knockdown zebrafish. Consistently, miR-451 partly compensates erythrocyte maturation defects in zebrafish with eif1axb knockdown and eIF1A(K56A) expression, supporting a role of eIF1A in miRNA-451 biogenesis in this model. Our results suggest that eIF1A is a novel component of the Ago2-centred RNA-induced silencing complexes (RISCs) and augments Ago2-dependent RNAi and miRNA biogenesis.
MicroRNAs (miRNAs) are ~22-nucleotide (nt) endogenous noncoding RNAs involved in gene expression regulation. Their genes are usually transcribed by RNA polymerase II or III. The resulting primary miRNAs contain characteristic hairpins, which are excised in the nucleus by Drosha/DGCR8 to yield the pre-miRNAs. Subsequently, the Exportin-5/Ran-GTP complex translocates the pre-miRNAs to the cytoplasm, where they are engaged by Dicer to form a RISC loading complex. The RISC loading complex includes TRBP and Ago2 (refs 7–9), while ADAR1 facilitates pre-miRNA loading. In the canonical miRNA biogenesis pathway, Dicer removes the terminal loop region to yield mature miRNA. Recent studies revealed that miR-451 is produced by an alternative Dicer-independent pathway, where pre-miR-451 is loaded directly onto Ago2 and sliced on the 3′-hairpin arm, as guided by the 5′-end of the hairpin, yielding a 30-nt cleaved species. A 3′-resection activity by PARN trims ~7 nt to produce the 23-nt miR-451 (ref. 17). However, the mechanisms of Dicer-independent miRNA biogenesis have remained elusive.

eIF1A binds to Ago2 in an RNA-independent manner. To identify which of the individual domain(s) of Ago2 interacts with eIF1A, we examined the interaction of recombinant—RNase A and Benzonase-treated—L1,PAZ, MID and PIWI domains of Ago2 with gluthatione S-transferase (GST)-tagged eIF1A in pull-down assays. RNA-free eIF1A specifically interacted with the RNA-free MID domain (Fig. 2a; Supplementary Fig. 1a). To further characterize the interaction between human eIF1A fragments and Ago2, we screened recombinant GST-tagged eIF1A and eIF1A mutants (N-tail deletion (ND), C-tail deletion and GD, Supplementary Fig. 1b,c) for interaction with Ago2. GST-tagged full-length, ND, C-tail deletion (CD) and GD of eIF1A interacted with Ago2 but not the GST-tag alone, indicating that the eIF1A-GD is required and sufficient for the interaction with Ago2 (Fig. 2b).

Results

eIF1A interacts with Ago2. To identify new components of the protein networks that participate in Dicer-independent miRNA biogenesis and Ago2-mediated RNAi, we generated a human embryonic kidney 293 (HEK293) cell line that stably expresses Ago2 primarily involves the GD of eIF1A. Furthermore, when unlabelled RNA-free eIF1A was titrated to the RNA-free 15N-MID domain at molar ratio of 1:1, we observed broadening of the MID resonances indicative of direct binding between the two proteins (Fig. 2d). We verified that eIF1A does not bind to the SMT3-tag (strong signals in Fig. 2d). These NMR data demonstrate that eIF1A directly interacts with Ago2 in an RNA-independent manner.

Ago2 interaction does not impair eIF1A translation functions. On the basis of the observed spectral changes (Fig. 2c), we generated three eIF1A mutants: V55A, K56A and K67A. To test whether the mutants are folded, we recorded a transverse relaxation optimized spectroscopy–heteronuclear single-quantum coherence spectrum of eIF1A. The spectrum clearly shows that eIF1A is properly folded (Supplementary Fig. 2). To examine the roles of Val55, Lys56 and Lys67 residues of 15N-eIF1A-GD show broadening upon MID domain titration (Fig. 2c). These results suggest that the interaction of eIF1A and Ago2 primarily involves the GD of eIF1A. Furthermore, when unlabelled RNA-free eIF1A was titrated to the RNA-free 15N-MID domain at molar ratio of 1:1, we observed broadening of the MID resonances indicative of direct binding between the two proteins (Fig. 2d). We verified that eIF1A does not bind to the SMT3-tag (strong signals in Fig. 2d). These NMR data demonstrate that eIF1A directly interacts with Ago2 in an RNA-independent manner.

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RNA-binding-independent manner, the MID domain of Ago2 binds to the GD of eIF1A and that the interaction between eIF1A and Ago2 does not impair eIF1A functions in translation initiation.

**eIF1A promotes Ago2-mediated miRNA-guided RNAi in vitro.**

To examine whether eIF1A plays a role in Ago2-mediated RNAi, we employed a human HMGA2 3’-untranslated region (UTR) mutant that harbours a characterized native let-7 target site35 (Fig. 4a). This assay evaluates the dependence of eIF1A and its mutants on let-7a/Ago2-mediated cleavage of the target mRNA. In the RNAi in vitro assay reactions with added eIF1A or eIF1A mutants without Ago2, no cleavage product was observed, suggesting that the cleavage is Ago2 dependent (Fig. 4b). Interestingly, we found that high concentrations of eIF1A increased HMGA2 3’-UTR mutant cleavage, while eIF1A mutants (V55A, K56A and K67A) significantly reduced the level of cleavage products (Fig. 4b,c), indicating that eIF1A augments Ago2-mediated RNAi activities in vitro. To investigate the effects of eIF1A in Ago2-mediated miRNA-guided RNAi activities in cells, we used green fluorescent protein (GFP) reporters containing let-7-targeted sequences36. Because transient knockdown of eIF1A in HEK293 cells led to significant decrease of cell viability (Supplementary Fig. 3), we employed specific eIF1A–Ago2 interaction interference with eIF1A K56A. Increased eIF1A levels elevated let-7 RISC activity, while eIF1A (K56A) mutant compromised let-7 RISC activity (Fig. 4d,e). Elevated eIF1A and Ago2 decrease cellular GFP mRNA levels as determined by quantitative (q)PCR (Fig. 4f). In contrast, eIF1A K56A caused increased cellular GFP mRNA levels (Fig. 4f). These results suggest that eIF1A forms a complex with Ago2 and promotes Ago2-mediated RNAi in human cells in vitro.

**eIF1A promotes Ago2-mediated miRNA-guided RNAi in vivo.**

To further evaluate the roles of eIF1A on Ago2-mediated RNAi in an animal model, we employed zebrafish. Both zebrafish...
Figure 2 | eIF1A binds to the MID domain of Ago2 in a RNA-binding-independent manner. (a) Human eIF1A interacts with the MID fragment of human Ago2. Recombinantly expressed and Benzonase-treated Ago2 fragments and recombinantly expressed GST-eIF1A were used for GST pull-down analyses followed by western blotting with anti-His antibodies (IB, immunoblotting). The arrow shows that the MID fragment interacts with eIF1A. (b) The globular domain (GD) of eIF1A interacts with Ago2. Recombinantly expressed and Benzonase-treated GST, GST-eIF1A and mutants (ND, 25–114aa; CD, 1–114aa; GD, 25–114aa) were used for GST pull-down assays with cell lysates of HEK293 stably expressing Ago2. (c) 1H–15N transverse relaxation optimized spectroscopy (TROSY)–heteronuclear single-quantum coherence (HSQC) spectrum of 15N-eIF1A titrated with the SUMO-MID (432–575aa) of RNA-free-Sumo-MID leads to broadening of RNA-free 15N-eIF1A resonances (molar ratio of 1:1). Inserted frames show the broadening of resonances of residues from the MID domain, but not from SMT3-T ag (SUMO-tag), upon eIF1A titration. The titration of eIF1A (molar ratio of 1:1) results in broadening 1H–15N TROSY–HSQC crosspeaks coming from the 15N-MID domain, but not from 15N-Sumo (SMT3) tag. Inserted frames show the broadening of resonances of residues from the MID domain, but not from SMT3-Tag (SUMO-tag), upon eIF1A titration.

paralogues eIF1AXa and eIF1AXb interact with Ago2 (Supplementary Fig. 4a,b). eiflaxb mRNA level is much higher (>25-fold) than that of eiflaxa in the developing zebrafish (Supplementary Fig. 4c,d), therefore we focused on eiflaxb here. We employed a lower dosage of eiflaxa Morpholino (MO, 62.5 μM, 1 nl), which does not significantly affect zebrafish phenotypes at 18 h.p.f. (hours post fertilization) and 48 h.p.f. (Supplementary Fig. 4e,f). Previous studies reported that miR-126 targets c-Myb and suppresses c-Myb expression in zebrafish37. We next examined whether eIF1A plays a role in Ago2-mediated miR-126-guided c-Myb suppression in zebrafish at 28 h.p.f. Zebrafish treated with eiflaxb MO (62.5 μM, 1 nl) showed increased c-Myb in comparison with the control group (Fig. 4g). Zebrafish with treatment of eiflaxb MO (62.5 μM, 1 nl) plus human eIF1A (heIF1A, 1 μg μl−1 mRNA, 1 nl) presented decreased c-Myb levels compared with the group treated with only eiflaxb MO (62.5 μM, 1 nl). In contrast, zebrafish treated with human eIF1A K56A (1 μg μl−1 mRNA, 1 nl) and eiflaxb MO (62.5 μM, 1 nl) show increased c-Myb (Fig. 4g). In addition, heIF1A K56A increased c-Myb mRNA accumulation in zebrafish (Fig. 4h), indicating that interfering with eIF1A–Ago2 interaction impairs the miR-126-mediated suppression of c-Myb expression in zebrafish, even though the level of miR-126 remaining is not significantly decreased under these conditions (Fig. 4i). These results demonstrated that the eIF1A–Ago2 complex promotes miR-126 RISC activities in vivo. Overall, the above results demonstrate that eIF1A augments Ago2-mediated miRNA-guided RNAi both in vitro and in vivo.

eIF1A stimulates miR-451 biogenesis in vitro and in vivo. Ago2 is important for Dicer-independent generation of mature miR-451, a miRNA that regulates erythropoiesis14–16. We therefore investigated whether eIF1A is involved in Ago2-mediated DICER-independent miRNA biogenesis. In vitro analyses of eIF1A/Ago2-dependent cleavage of pre-miR-451 showed that reactions with eIF1A or eIF1A mutants, but without Ago2, displayed no pre-miR-451 cleavage (Fig. 5a). In the presence of Ago2, however, eIF1A enhances pre-miR-451 cleavage in a dose-dependent manner (Fig. 5a,b). In contrast, the point mutation of V55A, K56A or K67A impaired the activity of Ago2 in this assay (Fig. 5a,b). These results suggest that eIF1A forms a complex with
 Ago2 to facilitate pre-miR-451 cleavage. In northern blot assays, eIF1A mutants (V55A, K56A or K67A) in stable HEK293 cells consistently decreased the production or accumulation of mature miR-451 in comparison with wild-type eIF1A (Fig. 5c). Importantly, the level of miR-144—which is processed from the same precursor transcript as miR-451 (refs 14,15)—remained unchanged (Fig. 5c). These results suggest that eIF1A promotes Ago2-dependent miR-451 biogenesis in human cells in vitro. While the levels of miRNA-451 increased during development from 24 to 56 h.p.f. in wild-type zebrafish, elf1axb knockdown led to decreased miR-451 production (Fig. 5d). Human eIF1A (1 µM, 1 nl) could rescue miR-451 production in elf1axb knockdown zebrafish (Fig. 5d). In contrast, heIF1AK56A failed to rescue miR-451 production during zebrafish development (Fig. 5d), indicating that eIF1A–Ago2 interaction plays a role in miR-451 biogenesis in vivo. Importantly, elf1axb knockdown does not decrease miR-144 production, indicating that elf1A does not affect Ago2-independent miRNA production (Fig. 5d). These results demonstrated that elf1A forms a complex with Ago2 and augments Ago2-dependent miR-451 biogenesis in zebrafish.

**elf1A modulates miR-451-mediated erythrocyte maturation.** Recent studies have reported that miR-451 plays an important role in erythrocyte maturation in zebrafish15,38,39. Consistent with a role for elf1A in miR-451 biogenesis, injection of human elf1A mRNAs (1 µg µl⁻¹, 1 nl, n = 35 embryos), but not mRNAs encoding human elf1AK56A (1 µg µl⁻¹, 1 nl, n = 35), rescues the reduced haemoglobinized cells in zebrafish with elf1axb gene knockdown (Fig. 5e,f). Importantly, miR-451 (0.2 µg µl⁻¹, 1 nl, n = 31) partly compensated the decrease in haemoglobinized cells in zebrafish treated with elf1axb knockdown or heIF1AK56A (Fig. 5e,f). Furthermore, heIF1A (n = 120 erythrocytes), but not heIF1AK56A mutant (n = 145 erythrocytes) rescued erythrocyte maturation in zebrafish with elf1axb knockdown. Notably, miR-451 (0.2 µg µl⁻¹, 1 nl, n = 118 erythrocytes) rescued erythrocyte maturation delay caused by heIF1AK56A expression (Fig. 5g,h). These results demonstrate that elf1A modulates miR-451 production in zebrafish. Taken together, our data demonstrate that elf1A forms a complex with Ago2 and promotes Ago2-mediated miRNA-guided RNAi and Ago2-dependent Dicer-independent miRNA biogenesis.

**Discussion**

Ago2-mediated miRNA biogenesis and processing are essential for the development in eukaryotes. We found that elf1A directly binds to Ago2 in an RNA-independent manner, and plays a role in both Ago2-mediated miRNA-guided RNAi and miRNA biogenesis (Fig. 6). Using biochemistry and NMR analyses, we demonstrated that the MID domain of Ago2 binds to the GD of elf1A. elf1A promotes Ago2-mediated RNAi and miR-451 biogenesis in vitro and in vivo. elf1A augments erythrocyte maturation in zebrafish through mediating miR-451 surveillance. The previously unrecognized roles of elf1A in Ago2-mediated miRNA processes identified here provide insights into the understanding of the mechanisms of miRNA-guided RNAi and miRNA biogenesis.
Figure 4 | eIF1A augments Ago2-mediated RNAi. (a) Sequence of the human HMGA2 3′-UTR fragment mutant with two native let-7 target sites. Filled circles: Watson–Crick complementarity; open circles: non-Watson–Crick complementarity. Mutated nucleotides are shown in blue and wild-type nucleotides were underlined (to neutralize site 2 and to reduce self-complementarity). (b) In vitro effects of eIF1A on Ago2-mediated RNA cleavage. eIF1A or the indicated mutants were incubated with Ago2, synthetic let-7a and 5′-32P end-labelled HMGA2 3′-UTR mutants. RNAs were analysed by northern blot and autoradiographs analyses. (c) Statistical analyses of three independent in vitro assays in b. (d) Western blot assays of effects of eIF1A and eIF1A (K56A) mutant on GFP-let-7 reporter expression. Ago2, eIF1A or eIF1A (K56A) were transiently overexpressed in HEK293 cells stably expressing reporter blot and autoradiographs analyses. (e) Western blot assays of effects of eIF1A and eIF1A (K56A) mutant on GFP-let-7 reporter expression. Ago2, eIF1A or eIF1A (K56A) were transiently overexpressed in HEK293 cells stably expressing reporter GFP-let-7. (e) The expression ratio between tubulin and GFP reporter in the same gel were calculated. Intact (100%) RISC activity was considered in control group in g, group in h and group in i. T otal RNAs were extracted from 1 nl) plus heIF1A (K56A) mutant (mRNA, 1 μg μl−1, 1,000 pl) and heIF1A (K56A) mutant (mRNA, 1 μg μl−1, 1,000 pl). Proteins were extracted from >10 zebrafish per group. (f) Relative c-Myc mRNAs in each group in g by qPCR. Total RNAs were extracted from >10 zebrafish per group. (i) Taqman PCR assays of relative zebrafish miR-126 (dre-miR-126) in each group in g. Total RNAs were extracted from >10 zebrafish per group. In all statistical comparisons, three independent experiments were performed (mean ± s.d., n = 3 experiments, Student’s t-test). ***P < 0.01.

Interestingly, point mutation of K56A in eIF1A leads to dissociation of eIF1A–Ago2 interaction. The eIF1A (K56A) mutant does not impair translation initiation. In contrast, eIF1A(K56A) impairs Ago2-mediated miRNA-guided RNAi and Ago2-dependent miR-451 production in vitro and in zebrafish in vivo. Consistently, miR-451 partly reverts reduced haemoglobinization and delayed maturation of erythrocytes in elfaxb knockdown zebrafish with overexpressed heIF1A (K56A). These data demonstrate that eIF1A–Ago2 interaction promotes Ago2-mediated RNAi and miRNA production but does not compromise eIF1A functions in translation initiation.
Figure 5 | eIF1A augments miR-451 biogenesis. (a) In vitro activity of eIF1A in Ago2-mediated cleavage of 5′-32P end-labelled synthetic pre-miR-451. RNAs were analysed by northern blotting and autoradiographs analyses. (b) Statistical analyses of the data in a. Data of three independent experiments were shown (mean ± s.d., n = 3, Student’s t-test). (c) Northern blot assays show the effects of eIF1A mutants on miR-451 biogenesis in HEK293 cells. Total RNAs were extracted from stable HEK293 cells expressing Flag-eIF1A and Flag-eIF1A mutants. Signals show mature miR-451 and miR-144 levels with U6 as a loading control. (f) Northern blot assays show the effects of eIF1A(K56A) mutant on miR-451 generation in the developing zebrafish. Total RNAs were extracted from zebrafish with/without treatments of control MO, eif1axb MO (62.5 μM, 1 nl), eif1axb MO (62.5 μM, 1 nl) plus heIF1A (mRNA, 1 μg μl−1, 1 nl), eif1axb MO (62.5 μM, 1 nl) plus heIF1A (K56A) (mRNA, 1 μg μl−1, 1 nl). (e) Haemoglobin (brown) was visualized by the oxidation of o-dianisidine (o-das) in zebrafish at 48 h.p.f. Haemoglobinized cells accumulate in wild-type (group I) and are reduced in eif1axb MO-injected embryos (group II). Human eIF1A, but not K56A mutant (group III, with less haemoglobinized cells), rescues haemoglobinized cells in eif1axb MO-injected zebrafish, while miR-451 spares K56A caused rescue defects. Scale bar, 100 μm. (f) Percentage of embryos with normal haemoglobinized cells in zebrafish. Control group (n = 35), rescued erythrocyte maturation impaired by eif1axb MO injection, while miR-451 (n = 118) spares distribution difference in the heIF1A(K56A) mutant group. ***P < 0.01.
Recently, Ameres et al.\textsuperscript{10} Chehoui et al.\textsuperscript{14} and Cifuentes et al.\textsuperscript{15} have reported that recombinant Ago2 is sufficient for slicer activity with large quantities of RNA fragments in RNAi and miR-451 processing in vitro. These data elegantly evidenced that Ago2 is a central effector of miR-451 generation and RNAi processes. On the other hand, many studies demonstrated that the Ago2-centred RISC complex is necessary for efficient RNAi activity with long and/or structured mRNAs and miR-451 processing in vivo\textsuperscript{3,4,5}. It is possible that other components in RISC are needed to resolve the mRNA secondary structure, to scan the long and/or structured mRNAs for recognition of miRNA seed regions in low abundance and to generate mature miR-451. Currently, the component list of RISC is increasing. Here we show that eIF1A directly binds to Ago2 and augments miR-451 biogenesis and RNAi processes, suggesting that eIF1A is an important but previously unrecognized factor of RISC.

The MID domain of Ago2 is essential for miRNA docking in RISC-mediated RNAi and pre-miR-451 loading in Dicer-independent miR-451 biogenesis. Here, we found that eIF1A directly binds to the MID domain of Ago2, and eIF1A promotes miRNA-guided RNAi and miR-451 generation.

Our findings reveal that eIF1A directly interacts with Ago2 and augments Ago2-mediated miRNA-guided RNAi and Dicer-independent miRNA biogenesis. The newly identified eIF1A–Ago2 complex together with its functions in miRNA processes provides insights in understanding how Ago2 mediates miRNA processes in translation, development and diseases.

### Methods

**Plasmids, antibodies and reagents.** Constructs of human Flag-HA-Ago1(10820), Ago2 (10822), Ago3 (10823) and Ago4 (10824) were obtained from Addgene. The complementary DNA (cDNAs) of human eIF1A were PCR-amplified from Hek293 cDNAs. Human eIF1A was inserted into NotI/EcoRI sites of the pIREShex vector. Mutants of eIF1A: ND (25 → 144 aa, amino acid), CD (1 → 144 aa), GD (25 → 144 aa) were generated by PCR and inserted into NotI/EcoRI sites of the pIREShex vector encoding an N terminus with Flag-HA. Monoclonal anti-Flag (M8160) antibody (20 μl for the immunoprecipitation assay), monoclonal anti-histone-HA antibody (6E2, 2367S, 20 μl for the immunoprecipitation assay) and monoclonal anti-β-Gal antibody (#29565, 20 μl for the immunoprecipitation assay, 1:1,000 dilution for the western blot assay) and anti-β-Tubulin (2146S, 1:3,000 dilution for the western blot assay) were ordered from Cell signaling. Monoclonal anti-eIF1A antibody (Ab172623, 20 μl for the immunoprecipitation assay; 1:1,000 dilution for the western blot assay), monoclonal anti-human Ago2 antibody (ab57113, used to detect human and zebrafish Ago2, 1:1,000 dilution for the western blot assay, 20 μl for the immunoprecipitation assay) and polyclonal anti-actin (ab8001, for zebrafish actin detection with 1:1,000 dilution for the western blot assay) were bought from Abcam. Polyclonal anti-Ago1 (SAB400655, 1:1,000 dilution for the immunoprecipitation assay) and Ago3 (SAB420012, 1:1,000 dilution for the western blot assay) antibodies were purchased from Sigma. Polyclonal anti-Ago4 antibody (MABE139, 1:1,000 dilution for the western blot assay) was ordered from Millipore. RN-Bonuclease A was ordered from Sigma. Benzoxane nuclease was ordered from VWR. Hek293 (CRL-1573), HeLa (CCL-2) and T98G (CRL-1690) cells were obtained from ATCC. SilverXpress Silver Staining Kit was purchased from Invitrogen. The CellTiter-Glo Luminescent Cell Viability Assay Kit was bought from Promega.

**Immunoprecipitation, MS and western blot assays.** The stable HEK293 cell line expressing Flag-HA-Ago2 (as a control) or Flag-HA-Ago2 were lysed with lysis buffer containing 20 mM Tris HCl pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40, EDTA-free protease inhibitor (Roche) followed by 10 μl of Proteinase K (20 μg/ml for the immunoprecipitation assay) and 1 μl of RNase A (20 μg/ml for the immunoprecipitation assay) and 1 μl of RNase T1 (10 U/ml for the immunoprecipitation assay) and 1 μl of RNase T1, immobilized on Dyna beads. The purified proteins were analysed by silver staining and mass spectrometry (MS) via liquid chromatography-MS/MS on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Germany) equipped with a Thermo Fisher Scientific nanospray source, an Agilent 1,100 Series binary HPLC pump and a Famos autosampler. The spectral data were searched with SEQUEST against a database containing the human International Protein Index protein sequence database (http://www.ebi.ac.uk/PIR/) together with the reversed complement. eIF1A was transiently overexpressed in stable Hek293 cell lines expressing Ago1–4. The cell lysates were treated with 10 μg/ml of 1 ribonuclease A (25°C, 1 h). Immunoprecipitated proteins by anti-eIF1A monoclonal antibodies (Abcam, Ab172623, 20 μl for the immunoprecipitation assay) were analysed by western blotting with anti-Ago1 (1–4) by monoclonal anti-Ago2 antibodies, Abcam, ab57113, 1:1,000 dilution; polyclonal anti-Ago1/3, Sigma, SAB4200065, SAB4200112, 1:1,000 dilution; Ago4 Millipore, MABE139, 1:1,000 dilution; antibodies in immunoblotting.

**Expression and purification of eIF1A and Ago2 fragments.** The cDNA for the MID domain of human Ago2 (His-Sumo-tagged) was provided by Bhushan Nagar (McGill University). Human eIF1A and mutants of eIF1A-ND/C-D/GD were inserted into EcoRI/NotI sites of pGEX-4E-1 vectors with GST fusion at the N-terminal. Human Ago2 (full length) and fragments of L1 (172–237 aa), PAZ (227–357 aa), MID (432–575 aa) and P1 (590–816 aa) were inserted into NdeI/BamHI sites of a pET-6H2 vector. His-tagged proteins expressed in BL21 (DE3) cells were extracted by sonication with lysate buffer (50 mM Tris, 10 mM EDTA, 10% glycerol, 150 mM NaCl, pH 7.5) and then loaded onto a Ni-NTA-agarose resin. After extensive washing with a buffer (50 mM Tris, 10 mM EDTA, 10% glycerol, 150 mM NaCl, pH 7.5), the resin was incubated with 10 μg/ml of 1 ribonuclease A (25°C, 1 h). The proteins were eluted with a buffer (100 mM imidazole, 50 mM Tris, 10 mM EDTA, 10% glycerol, pH 7.5). The purified proteins were analysed by silver staining and mass spectrometry (MS) via liquid chromatography-MS/MS on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Germany) equipped with a Thermo Fisher Scientific nanospray source, an Agilent 1,100 Series binary HPLC pump and a Famos autosampler. The spectral data were searched with SEQUEST against a database containing the human International Protein Index protein sequence database (http://www.ebi.ac.uk/PIR/) together with the reversed complement. eIF1A was transiently overexpressed in stable Hek293 cell lines expressing Ago1–4. The cell lysates were treated with 10 μg/ml of 1 ribonuclease A (25°C, 1 h). Immunoprecipitated proteins by anti-eIF1A monoclonal antibodies (Abcam, Ab172623, 20 μl for the immunoprecipitation assay) were analysed by western blotting with anti-Ago1 (1–4) by monoclonal anti-Ago2 antibodies, Abcam, ab57113, 1:1,000 dilution; polyclonal anti-Ago1/3, Sigma, SAB4200065, SAB4200112, 1:1,000 dilution; Ago4 Millipore, MABE139, 1:1,000 dilution; antibodies in immunoblotting.

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**Figure 6** Schematic diagram for eIF1A functions in Ago2-dependent miR-451 biogenesis, RNA interference and erythrocyte maturation in zebrafish. The primary miRNA (priRNA) is cleaved by Drosha-DGCR8 pathways to generate pre-miRNA within the nucleus. Exportin-5 in complex with Ran-GTP exports the pre-miRNA to the cytoplasm, where the pre-miRNA is bound by Dicer to form a RISC loading complex that includes Ago2. In the canonical miRNA biogenesis pathway, Dicer removes the terminal loop region to yield the mature miRNA. The pre-miR-451 is loaded directly onto Ago2 and sliced on the 3′-hairpin arm. The miR-444 biogenesis is Dicer dependent. The globular domain (GD) of eIF1A directly binds to the MID domain of Ago2 and forms an eIF1A–Ago2 complex promoting Ago2-mediated RNAi and miR-451 biogenesis. The MID domain of Ago2 bends to the GD of eIF1A and does not impair eIF1A functions in translation initiation. The 5′-end of the guide strand of miRNA–miRNA* duplex is docked onto a pocket with residues mainly from the MID domain. The long and structured mRNAs are scanned by RISCs to recognize seed regions in mRNAs. After a perfect or imperfect miRNA–miRNA* duplex is docked onto a pocket with residues mainly from the MID domain, eIF1A promotes Ago2-mediated RNAi and miR-451 biogenesis.
350 mM NaCl, 10 mM imidazole, pH 8.0, 20 μM Benzonase nuclelease, 10 μg ml⁻¹ RNase A, 100 μg ml⁻¹ lysozyme, 2% NP-40, 0.05% β-mercaptoethanol and protease inhibitor cocktail followed by Ni-agarose resin purification. Tagged proteins expressed in BL21(DE3) cells were extracted by MagneGST Protein Purification System (Promega) or Pierce Glutathione Magnetic Beads (Thermo Scientific).

**GST pull-down assays.** Escherichia coli-expressed GST-tagged human eIF1AX (known as eIF1A), or GD (25 μg of expression constructs) were purified with anti-HA antibodies and Dyna beads (Invitrogen) from HEK293 HA-Ago2, HA-eIF1A and HA-eIF1A mutant (V55A, K56A and K67A) proteins expressing His-tagged L1, PAZ, Sumo-MID, PIWI domains and Sumo, which were purified by Ni-NTA resin, were performed GST pull-down assays with E. coli expressed GST-eIF1A. Western blot assays were performed with anti-His monoclonal antibodies (Cell Signaling, #2366S, 1:1,000 dilution).

**Nuclear Magnetic Resonance (NMR) titrations.** BL21(DE3) bacterial cells harbouring His-SMT3, His-SMT3-tagged MID (425 ± 372) and His-tagged eIF1A were grown in either LB or minimal media containing 15N ammonium chloride. The resulting cell pellets were lyzed by sonication or French press with lysis buffer (50 mM Tris, 350 mM NaCl, 10 mM imidazole, pH 8.0, 20 μM Benzonase nuclelease, 10 μg ml⁻¹ RNase A, 100 μg ml⁻¹ lysozyme, 2% NP-40, 0.05% β-mercaptoethanol and protease inhibitor). The soluble proteins were loaded onto a Ni-agarose resin (Qiagen) and proteins were eluted with Ni-elution buffer (50 mM Tris, 350 mM NaCl, 350 mM imidazole, pH 8.0). Purified proteins in Ni-eluion buffer were further purified by size-exclusion chromatography using a S75 column and eluting with HEPES buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1 μg ml⁻¹ lysozyme, 2% NP-40, 0.05% β-mercaptoethanol) followed by Ni-agarose resin purification. GST-tagged human eIF1AX (K56A) and eIF1AX (K67A) were used in this study.

**miR-245 binding assay.** The interaction of miR-245 with eIF1A was investigated using a reporter construct harboring eIF1A and a T7 promoter sequence. This construct was transfected into 293 T cells, and after 48 h, the cells were harvested and RNA was extracted. The RNA was then subjected to Northern blot analysis to detect the presence of miR-245.

**miRNA expression and purification.** Total RNA was isolated from HEK293 cells stable cell lines expressing eIF1A or eIF1A mutant (K56A or K67A). The RNA was purified using the RNeasy Plus Mini Kit (Qiagen) and the purity was confirmed using a Nanodrop spectrophotometer. The miRNA was then reverse transcribed using the TaqMan Small RNA assay with the following sequences: let-7C, 5'-AGUUAUAUACUUACGUU-3' for let-7C; 5'-UACUAAUACUUACGUU-3' for Journey RNA. The qPCR mixture containing 1 μl of each with sense primers or antisense primers were separately performed (20 μM) and normalized to GlyC-5'-GATGTGACATAACCTCTTTGCACCATCGAAACACATTGC-3' for Glyc.

**Endogenous miRNA-guided RISC activity assays.** To read miRNA-guided RISC activity assays, the miR-245-3p (complementary to the 3'UTR of eIF1A) was used. The pCAG-miRNA-245 plasmid was co-transfected with pEGFP-C1 into 293 T cells. The interaction of miR-245 with eIF1A was investigated using a reporter construct harboring eIF1A and a T7 promoter sequence. This construct was transfected into 293 T cells, and after 48 h, the cells were harvested and RNA was extracted. The RNA was then subjected to Northern blot analysis to detect the presence of miR-245.

**Zebralight eIF1A knockdown and mRNA microinjection.** The wild-type A8/C32-strain zebralight were used in this study. All the experiments were conducted according to US National Institutes of Health guidelines for animal research and were approved by Harvard Medical Animal Committee. For the let-7 RISC activity assay, 2 μg of let-7 was transfected into HEK293 cells followed by 1.6 mg ml⁻¹ G418 selection for 2 weeks. The resistant colonies were propagated into stable cell lines. For the let-7 RISC activity assay, 2 μg of expression constructs were transfected into 5 × 10⁶ cells of the GFP reporter-stable cell lines in six-well plates and the cells were transferred to 10 cm plates after 24 h. After 36 h, the cells were subjected to cell-free conditions and the RNA was extracted using Trizol reagents. The control MO and 0.2 μg of eIF1A MO plus 1 μg ml⁻¹ human eIF1A or mutant miRNAs were injected into one-cell-stage zebrafish embryos. Total RNAs and proteins were isolated at 48 h.p.f. for the analyses of c-Myc proteins and mRNAs.

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25 mM MgCl2, 1 mM DTT and 0.5% (w/v) Triton X-100) and passed 6 × through a 27-gauge needle. Insoluble material was removed by a 3-min spin (micro-

**O-dianisidine staining.** O-dianisidine (Sigma) solution at 0.7 mg ml−1 was prepared in fresh ethanol and protected from exposure to light. The staining solution was prepared by mixing 2 ml of water, 2 ml of 0.7-mg ml−1 o-dianisidine solution, 0.5 ml of 100 mM sodium acetate and 100 μl of 3% hydrogen peroxide. Phenylthiohyluron (PTU)-treated 48-h-p.f. embryos were transferred to 12-well plates and 1 ml of the staining solution was added. Stained embryos were kept in the dark for 15 min, washed three times with 1 × PBS and fixed with 4% paraformaldehyde.

**May-Grünewald/Giemsma staining.** Erythrocyte collection from 20 zebrafish (56 h.p.f.) of each group was performed by cutting the zebrafish tails. Circulating erythrocytes were collected with 1 × PBS with 10% FBS with cytospin. The erythrocytes on slides were stained with May-Grünwald/Giemsa solutions (Polysciences), imaged and photographed using a Nikon 80i Upright Microscope. The pixel area of the nucleus and cytoplasm was quantified for 100–145 cells per sample in three independent experiments using ImageJ software, and the nucleocytoplasmic ratio was calculated for each cell.

**Image acquisition.** In o-dianisidine staining assays, stained embryos were imaged and photographed using a Leica M80 microscope with a Nikon D200 digital camera using an adjustable flash system. Erythrocytes with May-Grünwald/Giemsa staining were imaged using the Nikon 80i Upright Microscope in the Nikon Image Center at Harvard Medical School. Fluorescence and/or 4-O-dianisidine staining images were taken with a Nikon Ti Inverted Fluorescence microscope with Perfect Focus System in the Nikon Image Center at Harvard Medical School. Images of zebrafish embryos were taken by a Leica M80 microscope using a Nikon D200 digital camera.

**Statistical analyses.** All statistical experimental data are presented as mean ± s.d. (n = 3 or more). P value was determined by the Student’s t-test (tail = 2). ***P<0.01.

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NATURE COMMUNICATIONS | DOI: 10.1038/ncomms8194 | www.nature.com/naturecommunications

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Acknowledgements
We are grateful for support from NIH (grants CA068262 and GM047467) and the Agilent Foundation. For technical support and expertise, we thank the Nikon Imaging Center and the Institute of Chemistry and Cell Biology of Harvard Medical School, Charles Richardson, Yang Shi, Steven Elledge, Leonard Zon and Stephen Buratowski. We thank Qikai Xu for the helpful discussion during the early stage of the project.

Author contributions
T.Y., H.A. and G.W. designed and conceived the study. T.Y. performed experiments. H.A. and T.Y. performed HSQC assays. B.A. and T.Y. performed miRNA biogenesis and RNAi in vitro assays. H.S., T.Y. and C.G. performed zebrafish experiments. M.J. performed the mass spectrometric assay. T.Y. and H.H.Q. performed GFP reporter assays. E.P. and T.Y. analysed the interaction between eIF1A and Ago2 fragments. T.G. and T.Y. performed polysome profiling assays. All authors contributed to the final version of the paper. The manuscript was written by T.Y., H.A. and G.W.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yi, T. et al. eIF1A augments Ago2-mediated Dicer-independent miRNA biogenesis and RNA interference. Nat. Commun. 6:7194 doi: 10.1038/ncomms8194 (2015).