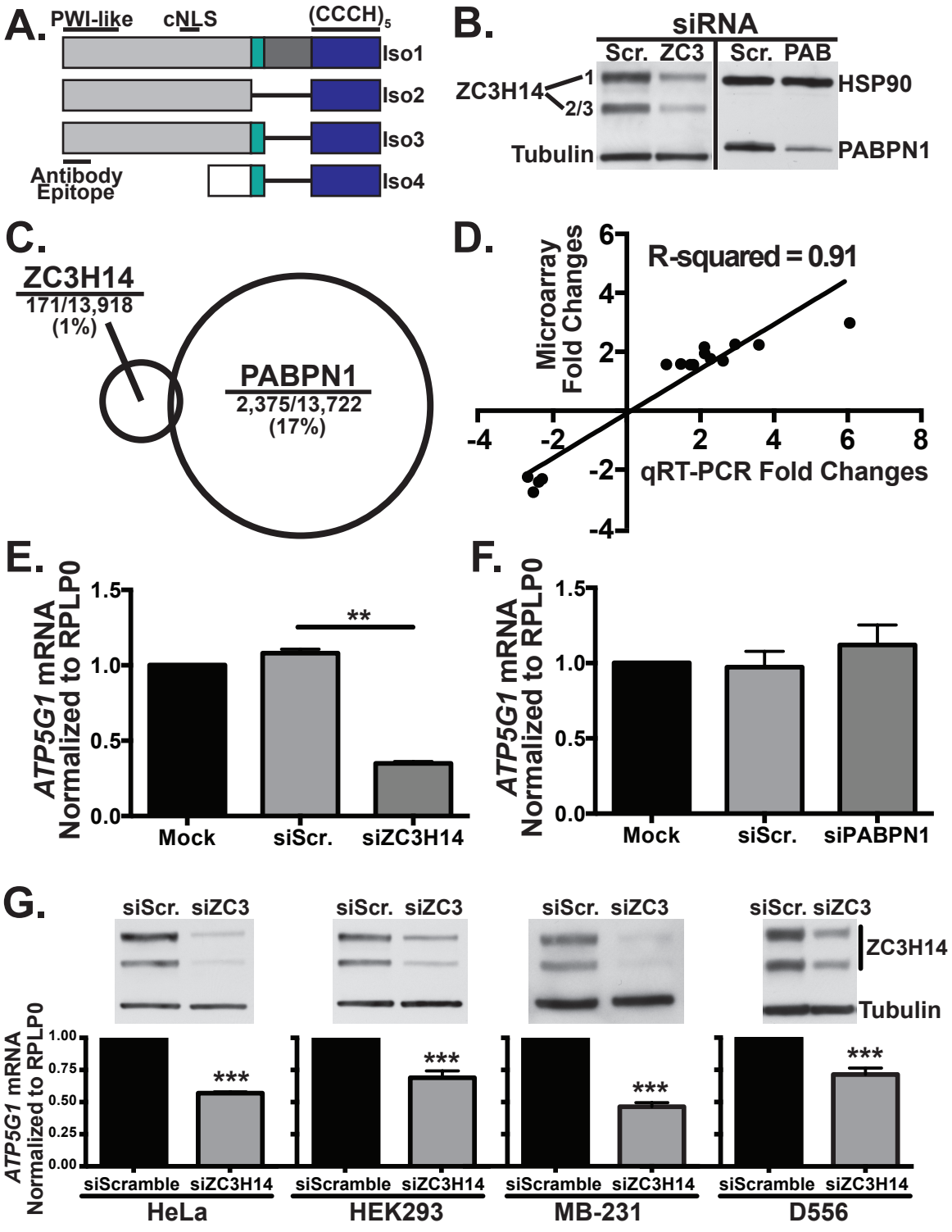
A.
PWI-like
cNLS
(CCCH)5
Iso1
Iso2
Iso3
Iso4
Antibody Epitope
B.
siRNA
Scr. ZC3
Scr. PAB
ZC3H14
1
2/3
Tubulin
HSP90
PABPN1
C.
ZC3H14
171/13,918
(1%)
PABPN1
2,375/13,722
(17%)
D.
R-squared = 0.91
Microarray Fold Changes
qRT-PCR Fold Changes
E.
ATP5G1 mRNA Normalized to RPLP0
**
Mock
siScr.
siZC3H14
F.
ATP5G1 mRNA Normalized to RPLP0
Mock
siScr.
siPABPN1
G.
siScr. siZC3
ZC3H14
Tubulin
ATP5G1 mRNA Normalized to RPLP0
***
siScramble siZC3H14
HeLa
HEK293
MB-231
D556

Figure 2.1: Knockdown of ZC3H14 decreases *ATP5G1* mRNA levels in all cell types examined. *A*) ZC3H14 is alternatively spliced to form at least four distinct protein isoforms (Iso1-4); three longer isoforms (Iso1-3) and a shorter isoform (Iso4). All isoforms contain the C-terminal CCCH5 zinc finger domain (blue) that confers RNA binding. Isoforms 1-3 differ from one another only in selective inclusion of exons 10-12 (teal and dark grey). These isoforms all contain an N-terminal Proline Tryptophan Isoleucine-like (PWI-like) fold as well as a predicted classical NLS (cNLS). Consistent with the presence of a cNLS, Iso1-3 are all localized to the nucleus at steady-state. ZC3H14 isoform 4 contains a distinct N-terminal exon (white). As the cNLS is absent from this isoform, Iso4 localizes to the cytoplasm at steady-state. The ZC3H14 antibody used in this study recognizes the N-terminal domain of isoforms 1-3 (Antibody Epitope). *B*) To assess knockdown, MCF-7 cells transfected with scramble (Scr.), ZC3H14 (ZC3), or PABPN1 (PAB) siRNA were subjected to immunoblot analysis with ZC3H14 or PABPN1 antibody and control antibodies to detect Tubulin and Heat Shock Protein 90 (HSP90). Robust knockdown of ZC3H14 and PABPN1 was observed with no effect on tubulin or HSP90 (controls). *C*) Total RNA isolated from MCF-7 cells transfected as in (*B*) was used for cDNA generation and hybridization to the Illumina BeadChip microarray platform. A schematic is shown indicating the relative number of transcripts that show a change (>1.5-fold) in steady-state level for each knockdown with size of circle representing fraction of transcripts impacted. Significance Analysis of Microarrays (SAM) analysis revealed that 171 out of 13,918 (~1%) of expressed transcripts in the transfected cells were affected (increased or decreased) by knockdown of ZC3H14 (101 increased and 70 decreased), whereas PABPN1 knockdown modulated 2,375 out of 13,722 (~17%) expressed transcripts (1,285 increased and 1,090 decreased). *D*) Fold-change values of select affected transcripts identified by the microarray analysis were plotted against fold-changes of the same select transcripts obtained by qRT-PCR analyses. Linear regression was used to determine the R2 value of 0.91, which represents a significant correlation between the results of both analyses and validates the effect on the transcripts analyzed. *E*) and *F*) Total RNA isolated from MCF-7 cells treated with mock transfection (Mock), scramble siRNA (siScr.), ZC3H14 (siZC3H14, Panel E) or PABPN1 (siPABPN1, Panel *F*) siRNA was used for cDNA generation and qRT-PCR analysis with transcript-specific primers to detect *ATP5G1* and the control *RPLP0* mRNA. Knockdown of ZC3H14 (Panel *E*), but not PABPN1 (Panel *F*), results in a significant decrease in *ATP5G1* steady- state mRNA levels. *G*) HeLa, HEK293, MB-231 and D556 cells (left to right) were transfected with scramble or ZC3H14 siRNA. Transfected cells were subjected to immunoblot analysis to confirm knockdown (top) with ZC3H14 and Tubulin (control) antibodies as well as qRT-PCR analysis (bottom) with *ATP5G1* and *RPLP0* (control) primers. Robust knockdown of ZC3H14 in each cell type resulted in a significant decrease in *ATP5G1* steady-state mRNA levels. Values represent the mean ± SEM for n=3 independent experiments. ** and *** represent $p \leq 0.01$ and $p \leq 0.001$, respectively.

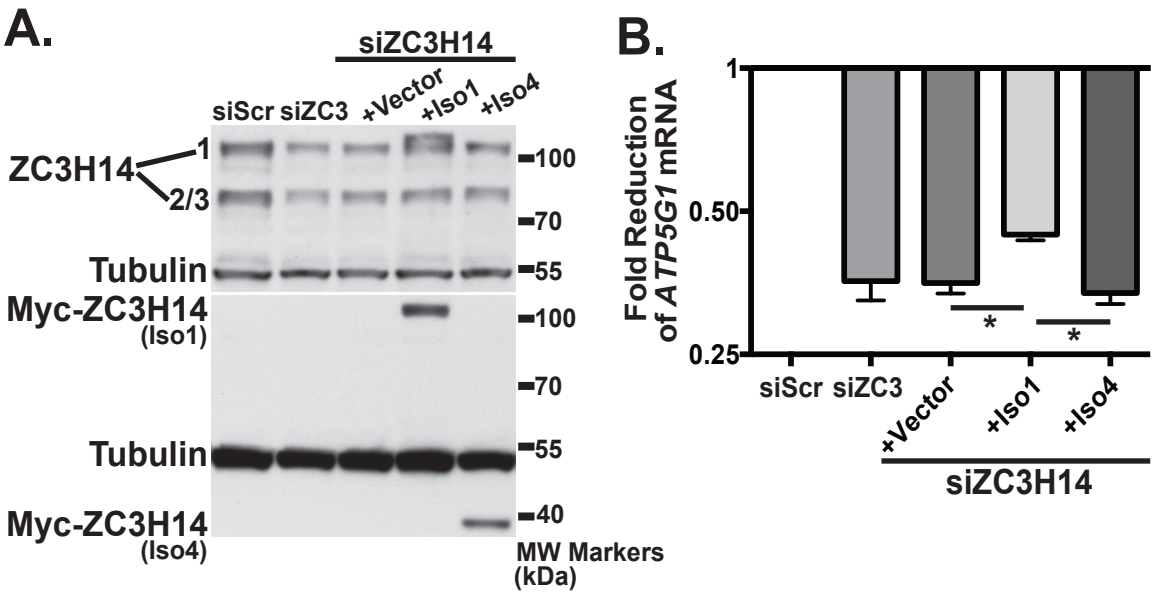

Figure 2.2: Re-expression of ZC3H14 isoform 1 restores *ATP5G1* transcript levels. To rescue the effect of ZC3H14 knockdown on *ATP5G1* mRNA levels, MCF-7 cells were transfected with either scramble (siScr.) or ZC3H14 (siZC3) siRNA alone or co-transfected with ZC3H14 siRNA and pcDNA3 (Vector), Myc-tagged ZC3H14 Isoform 1 (Iso1), or Myc-tagged ZC3H14 Isoform 4 (Iso4) for 48 hours. The Myc-tagged ZC3H14 constructs harbor silent mutations in the ZC3H14 siRNA-targeting regions and are therefore refractory to siRNA knockdown. Transfected cells were subjected to immunoblot analysis (*A*) with ZC3H14, Tubulin (control) or Myc antibody and qRT-PCR analysis (*B*) with primers specific to *ATP5G1* and control *18s rRNA*. Scramble control values are set to 1.0 and fold-reduction of ATP5G1 mRNA is represented on a log2 axis. A significant rescue of *ATP5G1* mRNA upon re-expression of Myc-Iso1 but not –Iso4 is indicated by *, which represents $p \leq 0.05$. Values represent the mean ± SEM for n=3.

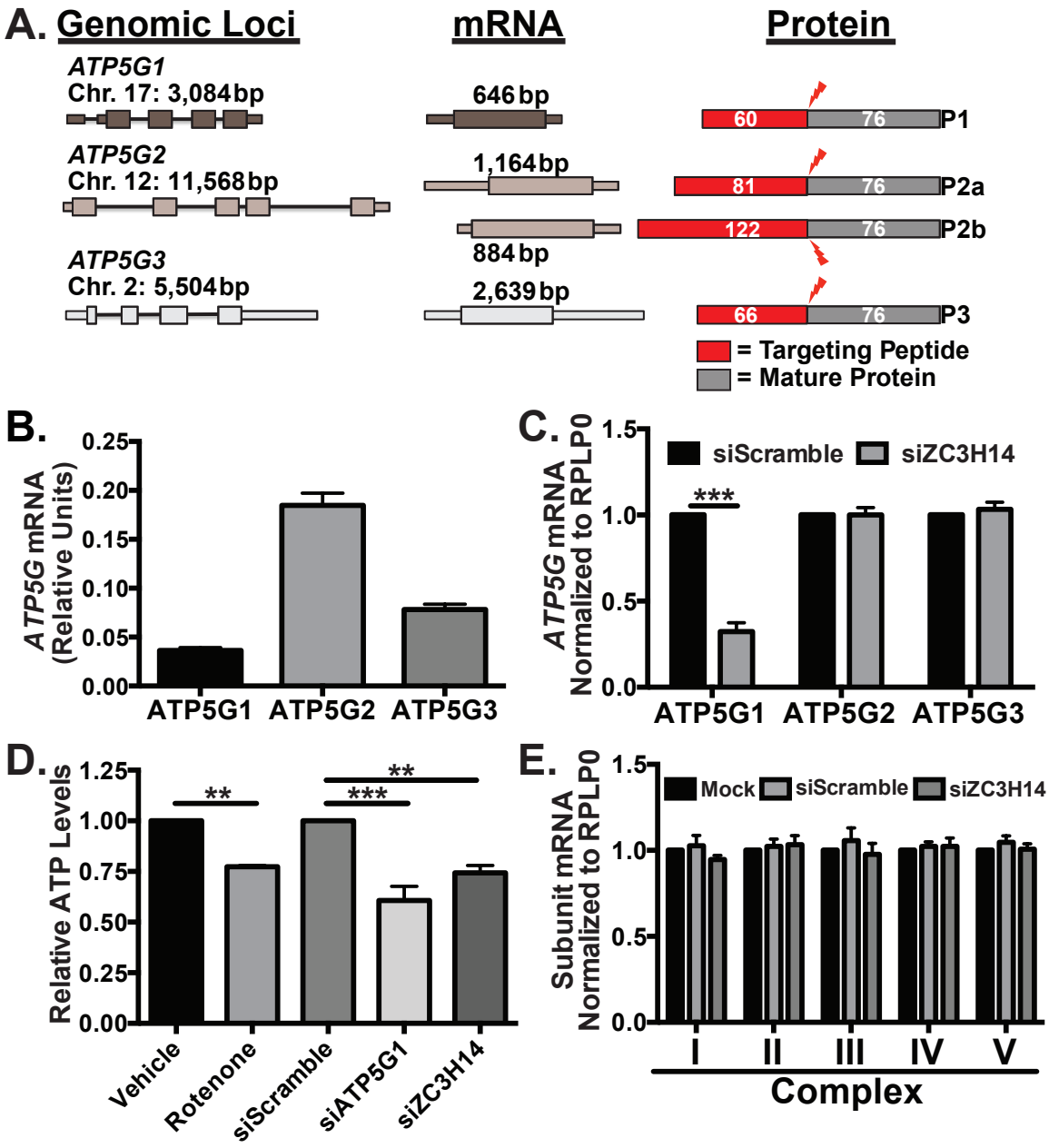

Figure 2.3: ZC3H14 modulates cellular ATP levels. *A*) The *ATP5G1*, *ATP5G2* and *ATP5G3* transcripts are transcribed from three separate genomic loci on chromosomes (Chr.) 17, 12 and 2, respectively. The *ATP5G1*, *-2*, and *-3* genes and their corresponding mRNAs have varying lengths (reported in base pairs, bp; thin bars=introns, thicker bands=UTRs, boxes=coding regions) and encode distinct protein products (P1, P2, and P3). *ATP5G2* is alternatively spliced to form two distinct mRNAs and subsequent protein products, P2a and P2b. The encoded protein products contain identical C-termini with variable N-terminal mitochondrial targeting peptides (red, with the number of amino acids indicated) that are cleaved (red lightning bolt) upon import into the mitochondria. The resulting mature protein products (dark grey) are completely identical in amino acid sequence (76 amino acids). *B*) Total RNA isolated from MCF-7 cells was used for qRT-PCR analysis with primers specific to each of the *ATP5G* mRNAs. The relative value of

each *ATP5G* mRNA was calculated by 2^-Ct and is reported as relative units. *C*) MCF-7 cells transfected with scramble control or ZC3H14 siRNA were subjected to RNA isolation and qRT-PCR analysis with primers specific to all three *ATP5G* mRNAs as well as the control transcript, *RPLP0*. Values are set to 1.0 for siScramble and normalized to RPLP0. Knockdown of ZC3H14 results in a specific and robust decrease in *ATP5G1* steady-state mRNA levels. *D*) To assess cellular ATP levels, cells treated with vehicle control, the electron transport chain inhibitor, rotenone, scramble siRNA, or siRNA targeting *ATP5G1* or *ZC3H14* were subjected to boiling water extraction and ATP level quantification using a luciferase-based assay. Cellular ATP levels are normalized to vehicle control or siScramble which are both set to 1.0 and plotted as relative ATP levels. ZC3H14 knockdown results in decreased cellular ATP levels similar to that observed with rotenone treatment or knockdown of ATP5G1. *E*) Cells treated with a mock transfection, scramble siRNA, or ZC3H14 siRNA were harvested and total RNA was used for qRT-PCR analyses. Primers specific to one representative nuclear-encoded mitochondrial mRNA from each OXPHOS complex as well as the control transcript, *RPLP0*, were used to assess any overall impacts of ZC3H14 on steady-state levels of transcripts encoding OXPHOS components I: *NDUFA4*, II: *SDHB*, III: *UQCRFS1*, IV: *CoxIV*, and V: *ATP5B*. Relative mRNA values for each OXPHOS mRNA from mock transfection are set to 1.0. Values represent the mean ± SEM for n=3 independent experiments. ** and *** represent $p \leq 0.01$ and $p \leq 0.001$, respectively.

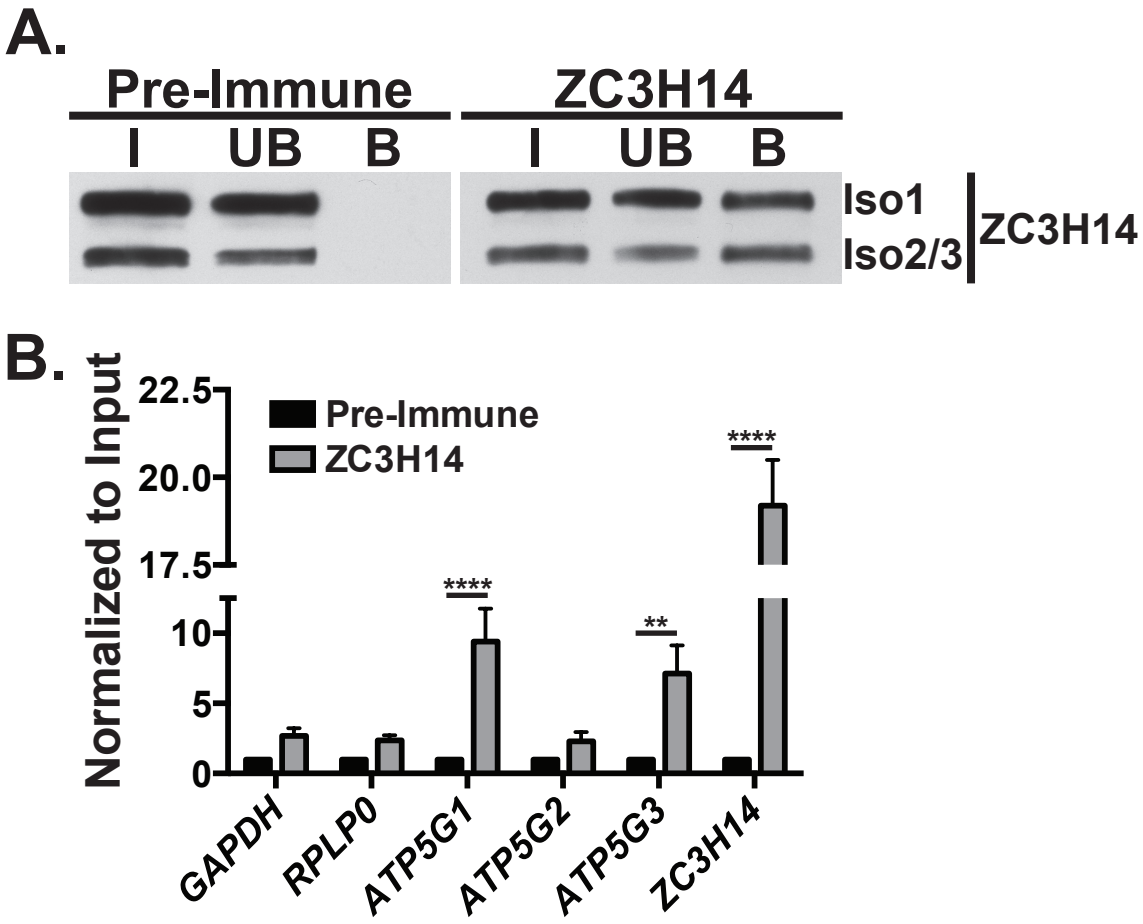

Figure 2.4: ZC3H14 binds to select mRNAs. Endogenous nuclear isoforms of ZC3H14 were immunoprecipitated from MCF-7 cells using either ZC3H14 antibody-bound protein A/G beads or rabbit pre-immune serum-coated beads. *A*) Proteins from the Input (I), unbound (UB) and bound (B) fractions were resolved on an SDS-PAGE gel and subjected to immunoblotting with ZC3H14 antibody. The nuclear ZC3H14 isoforms were detected in the ZC3H14-bound fraction but not the pre-immune bound fraction. *B*) RNA isolated from the ZC3H14 RNA-IP was subjected to qRT-PCR analyses with *GAPDH*, *RPLP0*, *ATP5G1*, *ATP5G2*, *ATP5G3* and *ZC3H14* primers. mRNA levels in the ZC3H14 bound fractions were normalized to input levels and then compared by fold-enrichment over pre-immune control. Significant enrichment of *ATP5G1*, *ATP5G3* and *ZC3H14* transcripts was observed with ZC3H14 IP. Values represent the mean ± SEM for n=3. ** and **** represent $p \leq 0.01$ and $p \leq 0.0001$, respectively.

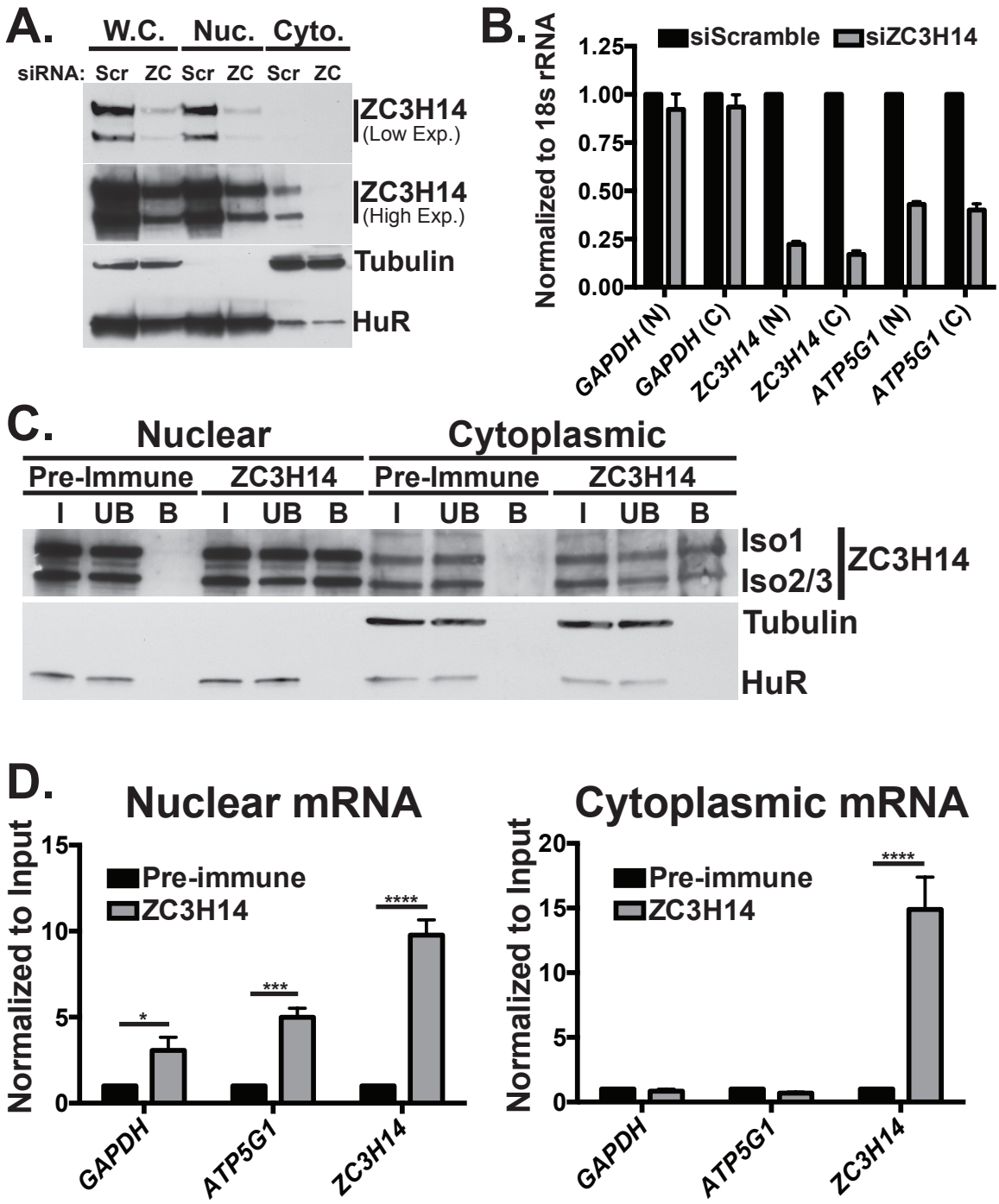

Figure 2.5: ZC3H14 binds to *ATP5G1* mRNA specifically in the nucleus. To determine if the effect on *ATP5G1* mRNA levels upon ZC3H14 knockdown is specific to a particular compartment, MCF-7 cells were transiently transfected with either Scramble (Scr.) or ZC3H14 (ZC) siRNA. Transfected cells were collected 48 hours later and subjected to nucleocytoplasmic fractionation. *A*) Protein from Whole Cell (W.C.), Nuclear (Nuc.) and Cytoplasmic (Cyto.) samples were subjected to immunoblot analysis with ZC3H14, Tubulin and HuR antibodies. As expected and consistent with clean fractionation, HuR and Tubulin display primarily nuclear and cytoplasmic localizations, respectively. Con

sistent with a previous study demonstrating steady-state nuclear localization of ZC3H14 in HeLa cells, we detect ZC3H14 primarily in the nucleus of MCF-7 cells. Robust knockdown of ZC3H14 in the whole cell and nuclear fractions is shown in the lower exposure blot (Low Exp.). A higher exposure (High Exp.) of the same blot demonstrates robust ZC3H14 knockdown in the cytoplasmic fraction as well. *B*) Total RNA isolated from samples in (*A*) was used for cDNA generation and qRT-PCR analysis with *GAPDH*, *ZC3H14* and *ATP5G1* primers. Knockdown of ZC3H14 resulted in a robust decrease of *ZC3H14* and *ATP5G1* steady-state mRNA levels in the nucleus and cytoplasm. To determine whether ZC3H14 interacts with *ATP5G1* mRNA in the nucleus and/or cytoplasm, MCF-7 cells were subjected to nucleocytoplasmic fractionation followed by RNA-IP, as in Figure 4. *C*) Proteins from the Input (I), unbound (UB) and bound (B) fractions were subjected to immunoblot analysis with ZC3H14, HuR and Tubulin antibodies. We achieve robust enrichment of ZC3H14 in each compartment. As expected, HuR and Tubulin are present primarily in the nuclear and cytoplasmic fractions, respectively. *D*) Total RNA isolated from the ZC3H14 RNA-IP in each compartment was subjected to qRT-PCR analysis with *GAPDH*, *ATP5G1* and *ZC3H14* primers. mRNA levels in the ZC3H14 bound fraction of each compartment were normalized to input levels and then compared by fold-enrichment over pre-immune control. Significant enrichment of *GAPDH*, *ATP5G1* and *ZC3H14* mRNAs was observed in the nucleus; however, *ZC3H14* was the only transcript enriched in the cytoplasmic samples. Data points represent the mean ± SEM for n=3 independent experiments. *, *** and **** represent $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.0001$, respectively.

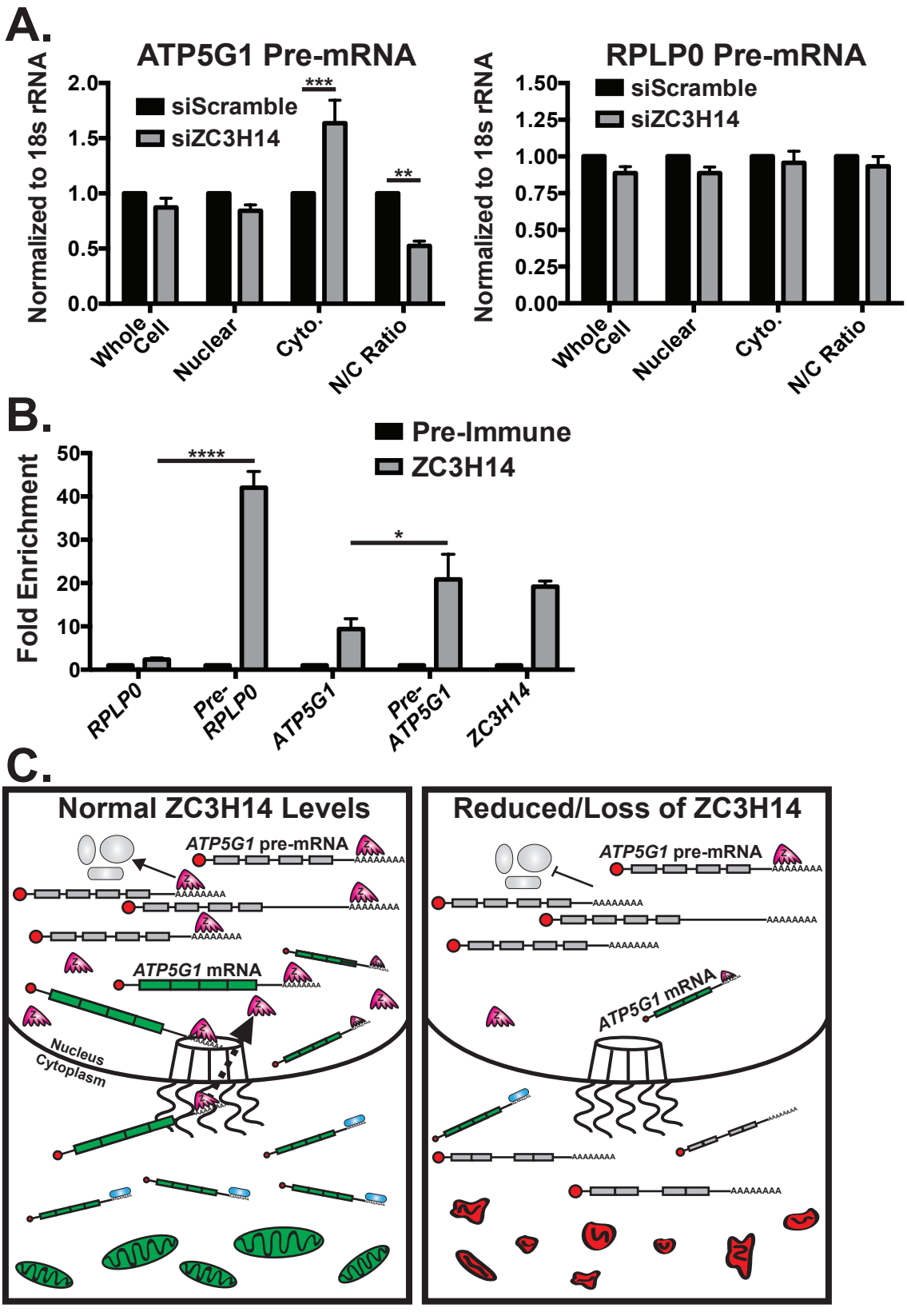
A.
ATP5G1 Pre-mRNA
Normalized to 18s rRNA
siScramble
siZC3H14
***
**
2.0
1.5
1.0
0.5
0.0
Whole Cell
Nuclear
Cyto.
N/C Ratio
RPLP0 Pre-mRNA
Normalized to 18s rRNA
siScramble
siZC3H14
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Whole Cell
Nuclear
Cyto.
N/C Ratio
B.
Fold Enrichment
Pre-Immune
ZC3H14
****
*
50
40
30
20
10
0
RPLP0
Pre-RPLP0
ATP5G1
Pre-ATP5G1
ZC3H14
C.
Normal ZC3H14 Levels
ATP5G1 pre-mRNA
ATP5G1 mRNA
Nucleus
Cytoplasm
Reduced/Loss of ZC3H14
ATP5G1 pre-mRNA
ATP5G1 mRNA

Figure 2.6: ZC3H14 is involved in the pre-mRNA processing of *ATP5G1*. *A*) Total RNA isolated from cells in Figure 5A were subjected to qRT-PCR analysis with primers to detect *ATP5G1* (left) and *RPLP0* (right) pre-mRNA. Knockdown of ZC3H14 did not affect the nuclear steady-state levels of *ATP5G1* or *RPLP0* pre-mRNA; however, cytoplasmic *ATP5G1* pre-mRNA levels increased in the ZC3H14 knockdown samples. Cytoplasmic *RPLP0* pre-mRNA levels were unchanged in the same samples. The Nuclear:Cytoplasmic (N/C) ratio of *ATP5G1* pre-mRNA is significantly decreased upon ZC3H14 knockdown, likely due to the increased cytoplasmic levels of *ATP5G1* pre-mRNA. The N/C ratio of *RPLP0* pre-mRNA is unchanged upon ZC3H14 knockdown. *B*) RNA isolated from samples in Figure 4 were subjected to qRT-PCR analysis with *RPLP0*, *RPLP0* pre-mRNA (*Pre-RPLP0*), *ATP5G1*, *ATP5G1* pre-mRNA (*Pre-ATP5G1*) and *ZC3H14* primers. Consistent with the results from Figure 4B, we observe significant enrichment of *ATP5G1* and *ZC3H14* mRNAs in the ZC3H14 bound fraction. Interestingly, we observe significantly higher enrichment of *RPLP0* and *ATP5G1* pre-mRNA levels compared to their respective mature transcripts. *C*) Left: In cells with normal levels of ZC3H14 (Z; pink, five-fingered shape), ZC3H14 interacts with poly(A) tails throughout nuclear processing events to ensure the coordination of proper pre-mRNA (represented with grey exons and including introns) processing events as well as and to couple these events to export, resulting in the selective export of export-competent mRNPs (represented with green, spliced exons). ZC3H14 is likely removed during the process of export (black dotted arrow). The proper production and export of *ATP5G1* mRNA maintains a healthy pool of mitochondria (green ovals at bottom of image). Right: In cells with reduced ZC3H14 levels, post-transcriptional processing events are not properly coordinated, resulting in a decrease in the production of mature mRNA and an increase in improperly and/or incompletely processed pre-mRNAs in the cytoplasm and a disruption in normal mitochondrial morphology (red shapes at bottom of image). Data points represent the mean ± SEM for n=3 independent experiments. *, **, *** and **** represent $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively.

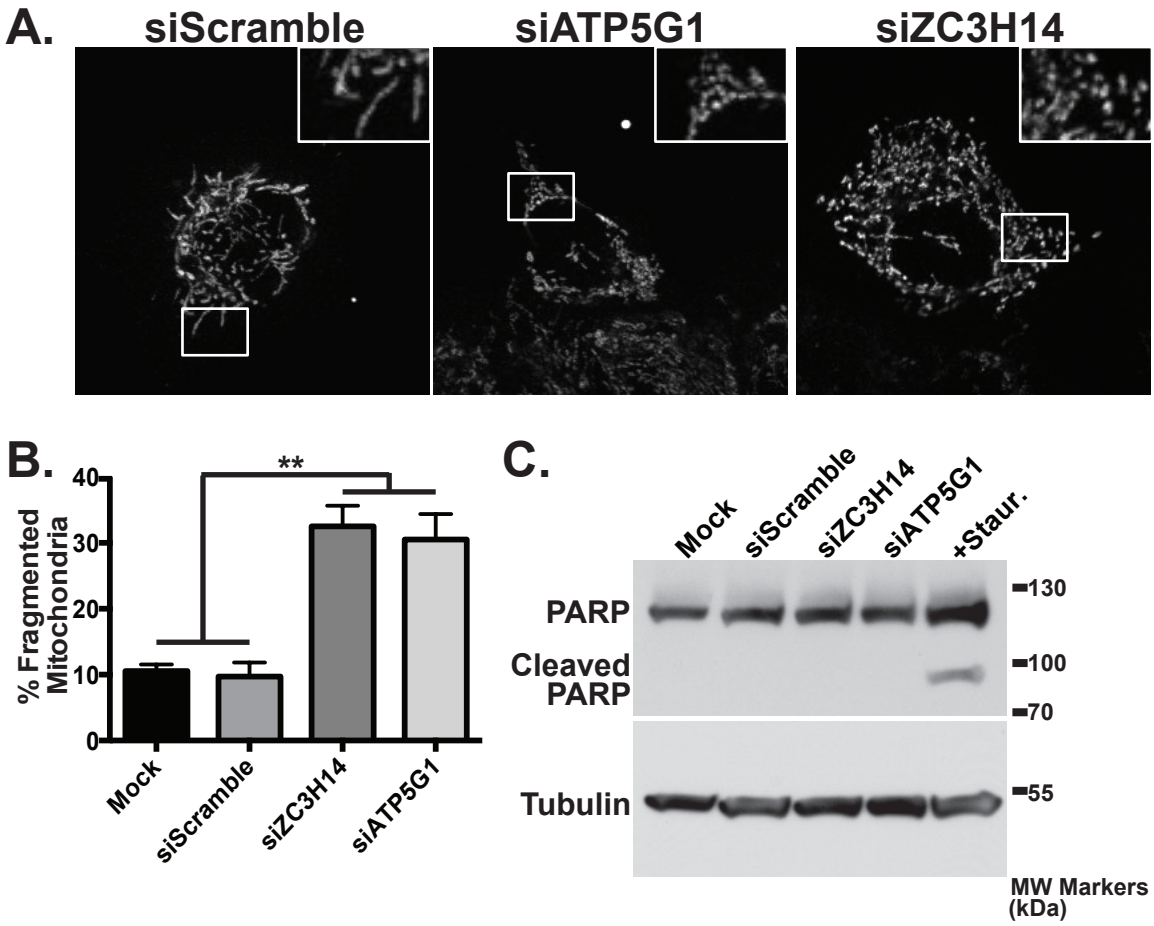

Figure 2.7: Knockdown of ZC3H14 results in fragmented mitochondria. *A*) MCF-7 cells transfected with either scramble (left), ATP5G1 siRNA (middle), or ZC3H14 (right) were fixed, permeabilized, and subjected to immunofluorescence with cytochrome C antibody. Insets are enlarged from the boxed regions of cells to better highlight mitochondrial morphology. Mitochondrial morphology in cells transfected with scrambled siRNA was indistinguishable from cells transfected with no siRNA (data not shown). *B*) Cells were scored for the presence of normal or fragmented mitochondria. Data is represented as a mean averaged from three independent experiments (N=304 for mock transfected cells, 311 for scrambled siRNA, 307 for ZC3H14 siRNA, 307 for ATP5G1 siRNA). The difference between control cells and either ZC3H14 or ATP5G1 cells was statistically significant. *C*) MCF-7 cells treated with mock transfection, scramble, ZC3H14, or ATP5G1 siRNA or the apoptotic inducer Staurosporine (Staur.) were subjected to immunoblot analysis with PARP or tubulin antibody. The presence of Cleaved PARP product only in the Staurosporine-treated samples suggests that the other cell populations are not undergoing apoptosis. Values represent the mean ± SEM for n=3 independent experiments. ** represents $p \leq 0.01$. Images are representative of n=3 independent experiments with 100 cells per experiment in each treatment group.