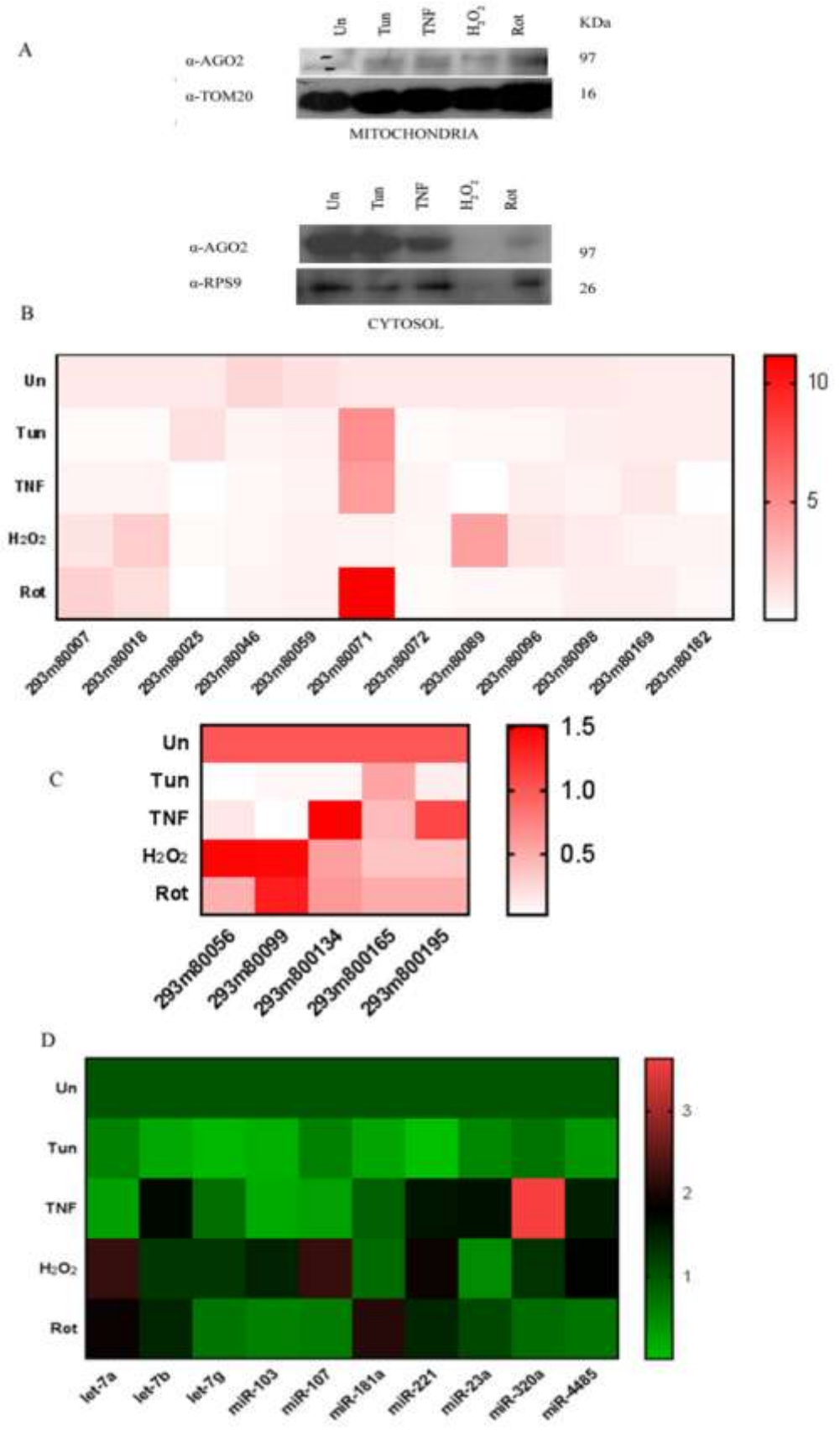
8. Association of miRNA with mitochondria is stress sensitive: miR-320a

Mitochondria are involved in numerous patho-physiological processes and its function is uniquely modulated in cell and stimulus specific conditions. The imported and associated proteome and RNAome depends on optimal, specialized and stimuli specific demands. Hence, the association of core miRISC component, Ago2, novel miRNAs and known miRNA in various cell death signals is determined. Further the association of miR-320a in TNF treatment and its role in mitochondrial function, cell death and tumorigenesis has been elucidated.

8.1 Association of miRNA is stress sensitive

The role of miRNA is important in regulation of target mRNA in narrow physiological range however, its implication in mitochondrial function is not understood. The cells were treated with ER stress inducer, tunicamycin; mitochondrial stress inducer, H_2O_2 and rotenone and pro-inflammatory cytokine, TNF-α and the level of miRNA in mitochondrial fraction was determined. The association of Ago2 increased in presence of TNF, ER stress (tunicamycin), H_2O_2 and rotenone treatments (Figure 8.1A). The association of novel miRNAs aligned to mitochondrial genome (Figure 8.1B), novel miRNAs with high count (Figure 8.1C) and 10 validated known miRNAs (Figure 8.1D) in stress was differential. The association of all selected miRNAs decreased in the presence of Tunicamycin, ER stress inducer. There was differential effect on the association of miRNAs in oxidative stress and mitochondrial stress. The enrichment of miR-293m80022, miR-293m80134 and miR-293m80195 increased upon TNF stimulation while the association of miR-293m80056 increased in H_2O_2 induced cell death. The association of miR-293m80099 increased in both H_2O_2 and rotenone treatment (Figure 8.1B). In tunicamycin induced cell death, the association of all 6 novel miRNA with high count decreased at mitochondria. These observations suggest the stimuli specific differential association dynamics of novel miRNAs at mitochondria. The physiological impact of these observations is intense area of research. Interestingly, the association of miR-320a remarkably increased upon TNF treatment. Hence, its impact was further characterised.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CYTOSOL
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2
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let-7a
let-7b
let-7g
miR-103
miR-107
miR-181a
miR-221
miR-23a
miR-320a
miR-4485

Figure 8.1. Association of Ago2 and miRNA with mitochondria alters in cell death. *The cells were treated with cell death inducers and mitochondria were prepared. A. The association of Ago2 in mitochondria and cytosols of these fractions was determined by western blotting using specific markers. The association of B. novel miRNA which aligned to mitochondrial genome, C. novel miRNAs with high count in small RNA libraries and D. 10 known miRNAs was determined by qPCR.*

8.2 Association of miR-320a at human mitochondria

The level of miR-320a significantly increased in mitochondrial fraction (Figure 8.1D) in the presence of TNF-α in HEK293 cells. The enrichment was also observed in mitochondrial fraction in the presence of TNF-α in other cancer cell lines, HeLa and MCF-7. We further analyzed its specific localization within mitochondria in the presence of TNF-α. The mitochondria were treated with RNaseA to determine if miR-320a is bound to outer membrane or oriented to mitochondrial matrix. Remarkable depletion of miR-320a (Figure 8.2A) was observed whereas 12S rRNA (Figure 8.2B) remained unchanged suggesting the association of miR-320a at outer membrane of mitochondria.

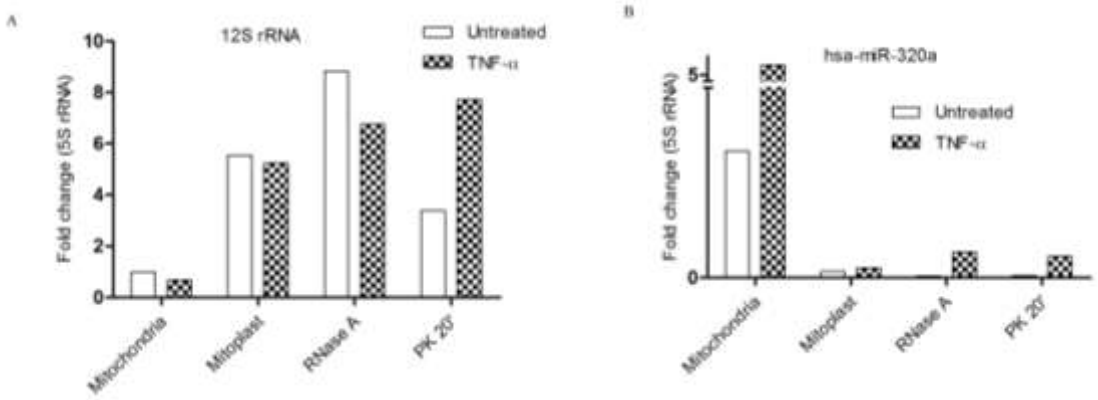

Figure 8.2. miR-320a associates with mitochondria in presence of TNF-α. *The association of miR-320a (A) and 12S rRNA (B) at mitochondrial surface was determined in HEK293 cells. The cells were treated with TNF-α and mitochondria were purified. The mitochondrial fraction was incubated with digitonin, RNase A, Proteinase K (PK) along with Triton-X 100. The mitochondria were washed, RNA isolated to determine miR-320a levels.*

Mitochondrial outer membrane anchors ribosomes and different RNA binding proteins involved in translation and RNA induced silencing complex like Ago2. Hence, we treated the mitochondria with Proteinase K. The level of miR-320a decreased in mitochondrial fraction in the presence of Proteinase K. To further confirm this, mitoplasts were prepared and its levels were monitored. MiR-320a level decreased in mitoplasts further confirming its association with RNA binding proteins on the outer mitochondrial membrane. 12S rRNA is specifically present inside the mitochondria hence each treatment showed high level suggesting integrity of mitochondria.

8.3 miR-320a regulates mitochondrial super complex-I assembly and function.

The translocation of mRNA/ncRNA to mitochondria is essential for its optimal function however the function of miRNA associated with mitochondria is not known. Hence, we further analyzed if mitochondrial associated miR-320a regulates mitochondrial functions.
The transfection of mimic decreased ATP levels, which were further reduced in the presence of TNF-α (Figure 8.3A). We further, measured its effect on mitochondrial membrane potential and ROS generation. The expression of mimic decreased the transmembrane potential which further decreased in the presence of TNF-α (Figure 8.3B). The transfection of miR-320a mimic increased ROS production which was further elevated in the presence of TNF-α (Figure 8.3C). The loss of membrane potential and elevated ROS is signature of mitochondrial ETC defect hence, we further studied its role in regulation of mitochondrial complex-I activity. In consonance to the above observation, the mitochondrial complex-I activity decreased in the presence of mimic which further decreased upon TNF-α stimulation (Figure 8.3D). To further confirm this, we performed BN-PAGE and in gel activity of mitochondrial complex-I. It was observed that miR-320a impairs mitochondrial super-complex-I. Interestingly accumulation of lower molecular sub-complexes of complex-I were observed. The activity of complex-I also decreased in the presence of miR-320a (Figure 8.4A). We further monitored if other complexes are also affected. Mitochondrial proteins were resolved by BN-PAGE and stained with coomassie brilliant blue. The protein level of mitochondrial Complex-I decreased whereas the level

of Complex-II, Complex-IV and Complex-V were unaltered while the levels of Complex-III increased in the presence of miR-320a mimic in HEK293 cells (Figure 8.4B). The mitochondrial genome encodes core subunits of mitochondrial Complex-I, hence we further determined its effect on the protein level of mitochondrial genome encoded subunits. MiR-320a was transfected and the cells were cultured in the presence of methionine homolog, AHA (Azido-Homo-alanine) in methionine depleted medium. The amount of AHA was determined by tagging with biotin using. The transfection of miR-320a mimic specifically decreased ND5, COI and ND3 whereas the level increased in presence of miR-320a inhibitor transfected cells (Figure 8.4C). These results suggest that miR-320a differentially regulates the mitochondrial encoded proteins, affects mitochondrial super complex assembly and function.

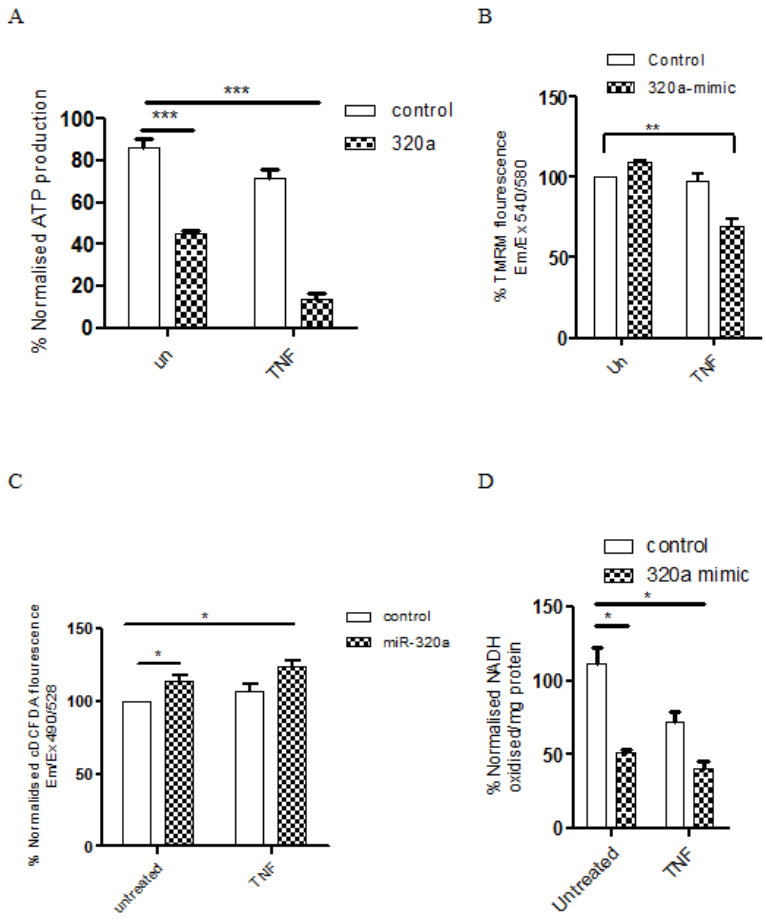

Figure 8.3. miR-320a modulates mitochondrial functions. *miR-320a was transfected in HEK293 cells, treated with TNF-α and ATP levels (A), transmembrane potential (B), ROS levels(C) and mitochondrial complex-I (D) were performed as described in methods.*

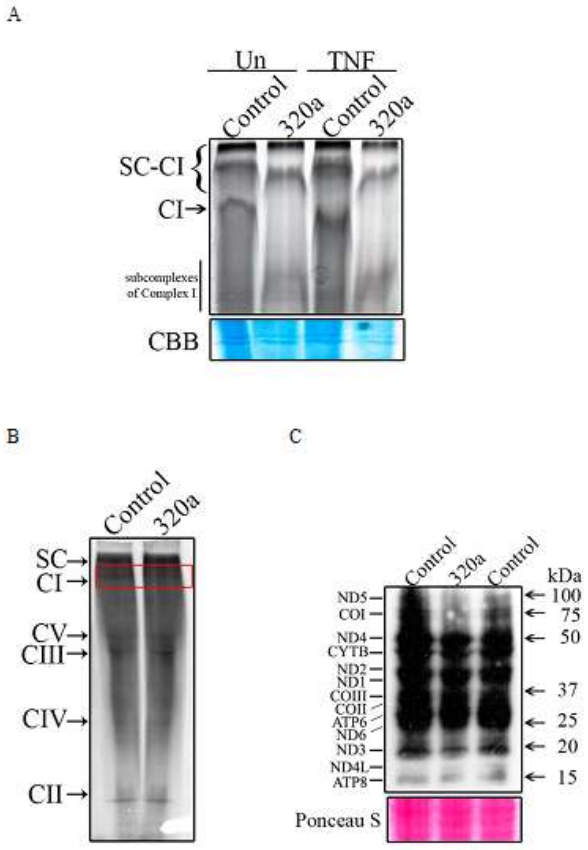

Figure 8.4. miR-320a regulates mitochondrial complex-I assembly. *miR-320a mimic was transfected in HEK293 and treated with TNF-α to determine in gel mitochondrial complex-I activity and super complex assembly (A), individual mitochondrial complexes (B) and mitochondrial genome encoded protein synthesis by methionine homologue incorporation assay (C).*

8.4 miR-320a regulates NDUFA10 at mitochondria

Previous reports suggest that decrease in ND3 and ND5 may not correspond to observed complex-I assembly defects (Lazarou et al., 2009). Hence, to further identify the core factor(s), we predicted the putative targets of miR-320a by StarBase using 5 different target prediction tools and clustered into meaningful groups using DAVID platform. The targets were clustered into pathways involved, OMIM disease, molecular functions, enriched tissue and cellular compartments. 59% of putative targets were involved in cancer and cell death pathways (Figure 8.5A) and highly enriched in brain and epithelial cells (Figure 8.5B). The putative targets included several nuclear encoded mitochondrial

proteins like EARS2, GLUD1, COX11, SDHC, SDHD, NDUFS1 and NDUFA10 (Figure 8.5C, Table 8.1).

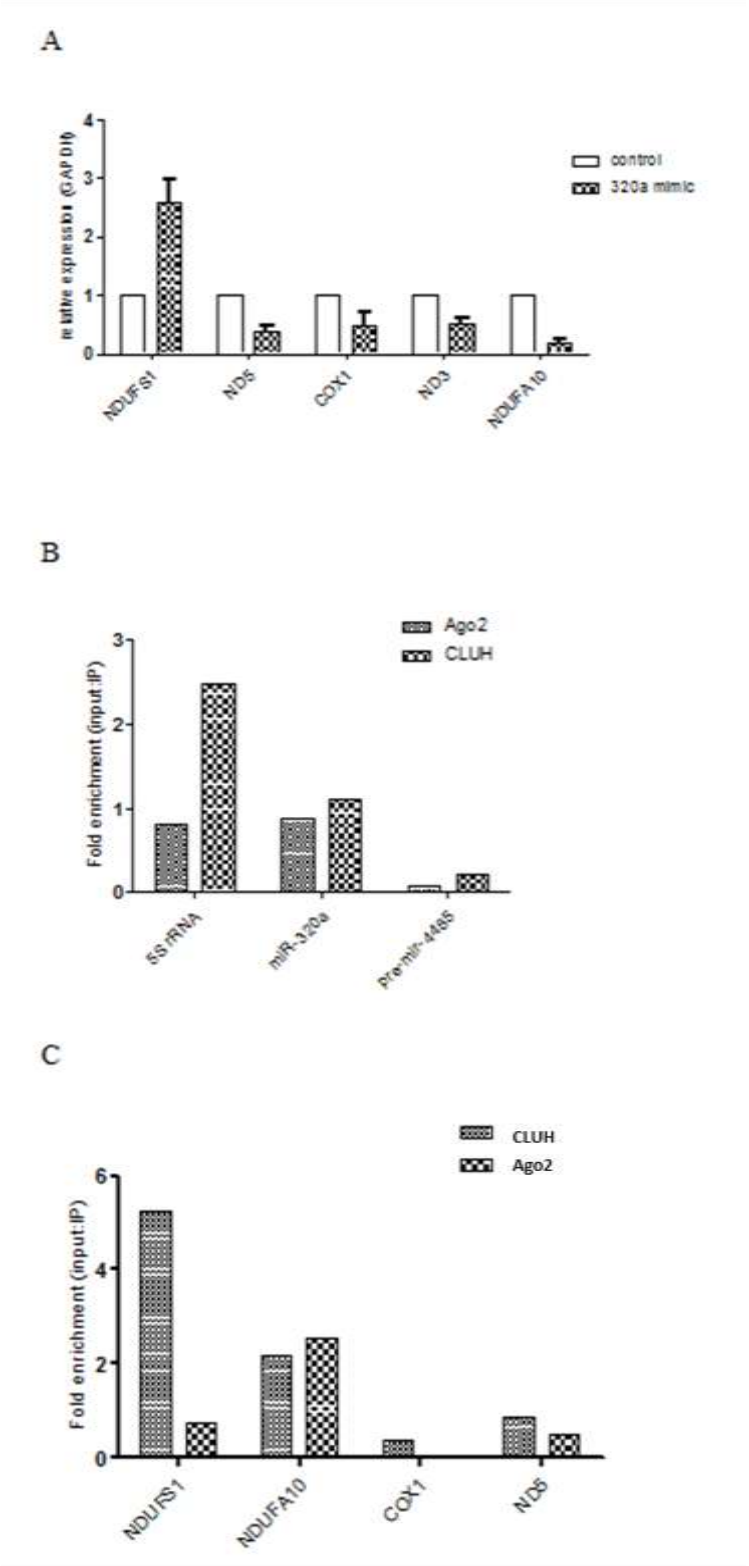

Figure 8.5. miR-320a regulate its target at mitochondria. *(A) The transcript levels of target was determined in HEK293 cells transfected with miR-320a mimic. The association of miR-320a (B) and the target transcripts associated with CLUH and Ago2 (C) at mitochondria was determined in HEK293 cells as described in methods.*

A recent report suggested the enrichment of nmRNAmp with RNA binding protein CLUH which further localized these transcripts on mitochondria. Hence, we further hypothesized that miR-320a may associate with and its cognate nmRNAmp with CLUH and Ago2 at mitochondria. The transcript level of NDUFS1, a subunit of N-module of complex-I, localized at the tip of hydrophilic arm towards matrix and NDUFA10 which is a component of Q module of hydrophobic arm at inner membrane, was determined. The transfection of miR-320a mimic decreased the level of NDUFA10 whereas the level of NDUFS1 increased (Figure 8.6A). Next, we determined which of these transcripts binds to CLUH and Ago2 by RNA immunoprecipitation assay. Flag tagged CLUH and Ago2 were transfected in HEK293 cells, mitochondria were isolated, Ago2 and CLUH were immuno precipitated using Anti-Flag beads, RNA isolated and analyzed. The level of miR-320a was enriched both in Ago-2 and CLUH immunoprecipiates (Figure 8.6B). We have previously shown that precursor of miR-4485 is not detected in mitochondrial fraction hence it was used as negative control. The level of pre-mir-4485 was not detected. Further, the level of the targets of miR-320a was analyzed in the immunopreciptiates of CLUH and Ago-2. Interestingly, NDUFA10 mRNA was enriched in both CLUH and Ago-2 immune precipitates. The enrichment of NDUFS1 mRNA was predominantly observed in CLUH as compared to Ago-2 IP (Figure 8.6C). This suggests that NDUFS1 and NDUFA10 are transported with CLUH at mitochondria, wherein miR-320a regulates NDUFA10 along with Ago2.

8.5 miR-320a regulates cell death and tumorigenicity

The evidences here strongly suggest that miR-320a regulates complex-I assembly and mitochondrial functions. Mitochondria are central executioner of cell death in different patho-physiological stimuli (Estaquier et al., 2012). Further, experiments were performed to test if miR-320a modulates cell death. The expression of mimic of miR-320a decreased

cell viability (Figure 8.7A). To further confirm the type of cell death, we monitored PARP cleavage, a marker of apoptotic cell death pathway. The transfection of miR-320a mimic showed increased level of 89kDa corresponding to cleaved subunit by caspases in both untreated and TNF-α as well as etoposide (DNA damage inducer) as compared to control (Figure 8.7B). This suggests the potentiation of apoptotic cell death hence we analyzed markers of apoptosis.

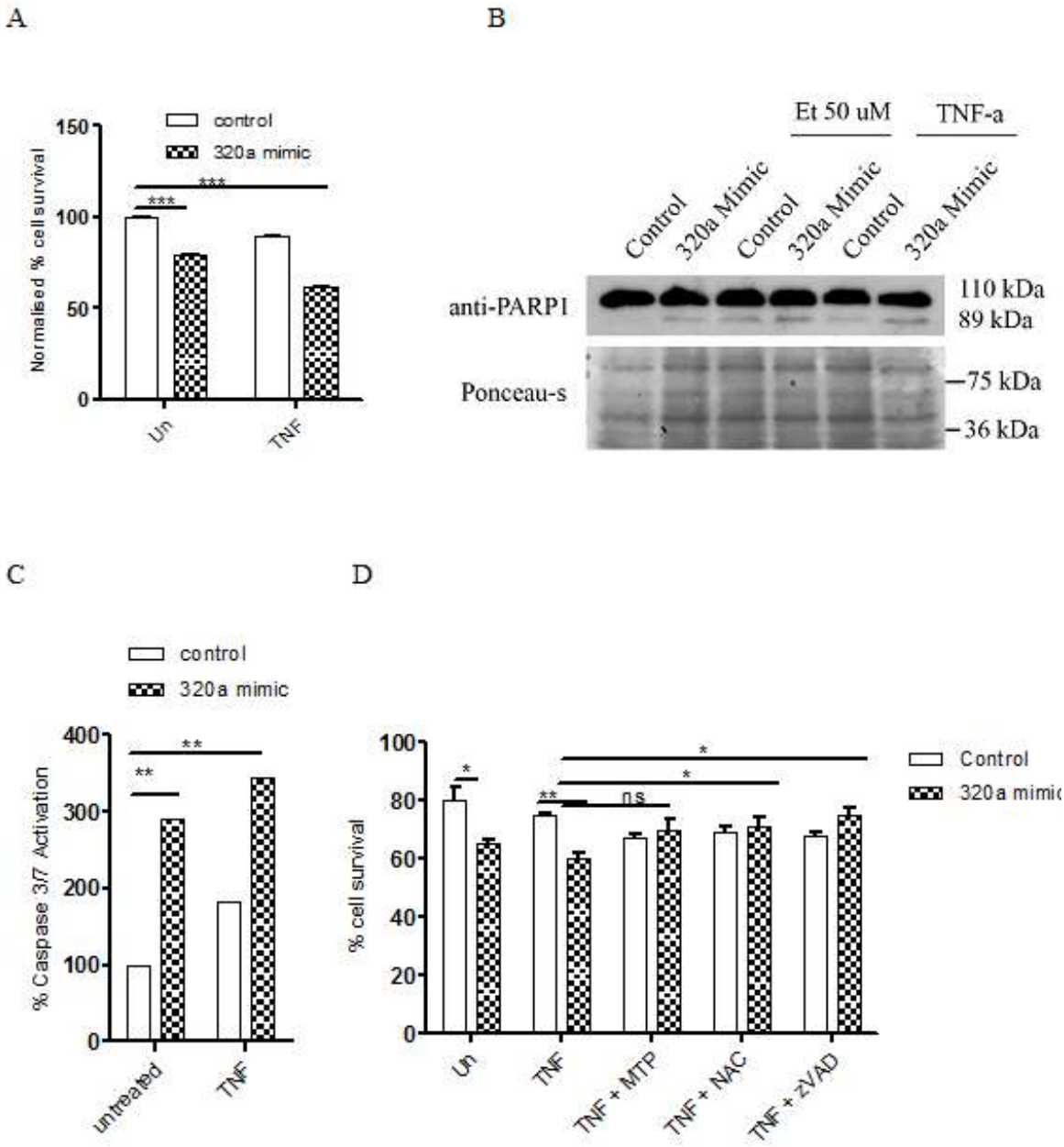

Figure 8.6. miR-320a potentiates ROS mediated cell death. *The mimic of miR-320a was transfected in HEK293 and cellular viability was assessed by MTT assay (A), PARP cleavage by western blotting (B) and Caspase 3/7 activity was determined using Caspase-GloR 3/7 Assay kit (C). The rescue of miR-320a induced cell death by ROS scavengers, NAC and Mito-Tempo (D) was performed* $^{*}P < 0.01$, $^{***}P < 0.0001$.

The cleavage of PARP is mediated by executioner caspases hence caspase-3/7 activity was monitored by luciferase assay. The transfection of miR-320a increased caspase 3/7activity (Figure 8.7C) as compared to control mimic transfected cells which further increased in the presence of TNF-α. As observed in above experiments, miR-320a mimic decreased mitochondrial Complex-I activity, increased ROS levels and activated apoptotic pathway, hence we co-treated cells with ROS scavengers, Mito-Tempo and N-acetyl cysteine (NAC). The treatment of cells with both ROS scavengers rescued miR-320a induced cell death upon TNF-α treatment (Figure 8.7D). This suggests that miR-320a regulates apoptosis by modulating mitochondrial functions.The metabolic reprogramming is one of the important adaptations of the tumor cells. Hence, we further analyzed if miR-320a act as tumor suppressor by modulating clonogenic and potential, migration ability. The transfection of miR-320a mimic decreased colony forming ability in cancer lines, MCF-7 both in absence and presence of TNF-α (Figure 8.8A, B). As described previously, miR-320a mimic elevated ROS levels and potentiated cell death by caspase activation, hence we tested clonogenic ability in the presence of ROS scavengers. The clonogenic ability was rescued in the presence of NAC. The mitochondrial dysfunctions suggest that cells might undertake glycolytic pathway as response mechanism which is expected to reverse the observed impact on tumorigenic assays. In contrast, the transfection of mimic decreased PFKM2, HK1 and PGAM1, markers of glycolytic pathway (Figure 8.8C). These results strongly suggest its role as a potential tumor suppressor. Mir-320a potentiates ROS mediated cell death; hence we determined the impact of ROS inhibition by NAC on miR-320a induced tumor suppression. The suppression of tumorigenic potential induced by miR-320a was rescued in the presence of NAC (Figure 8.8D).

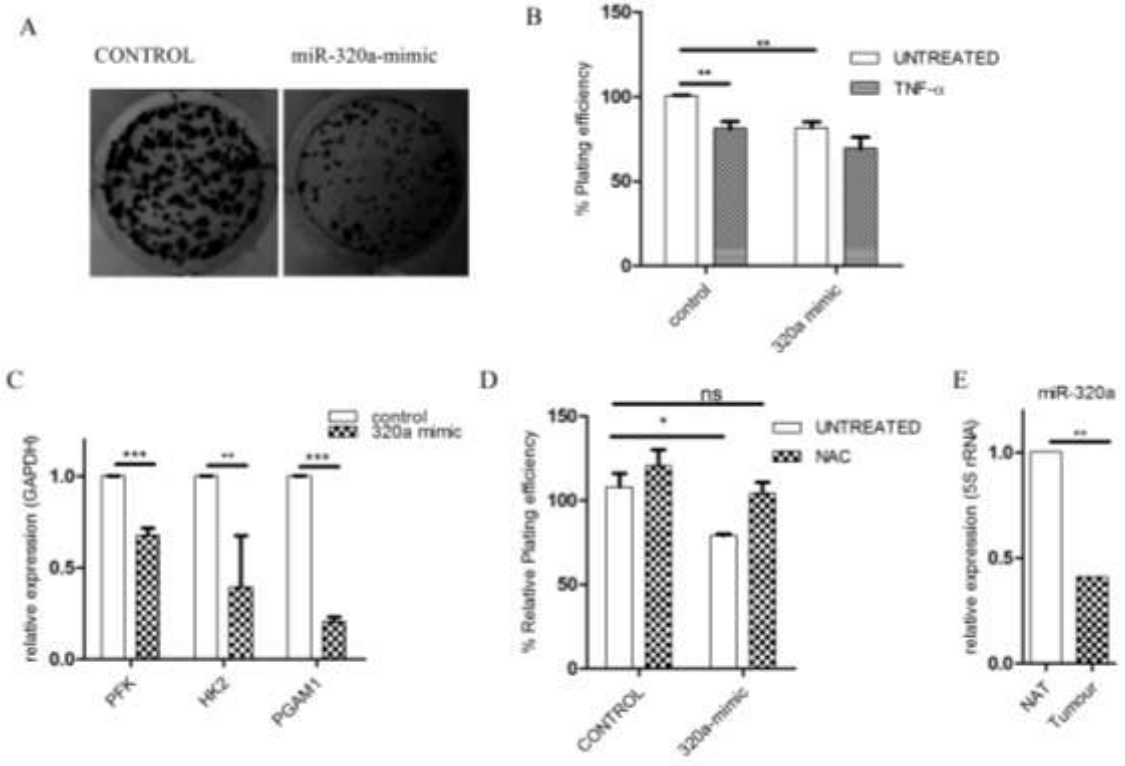

Figure 8.7. miR-320a negatively regulates ROS mediated tumorigenic potential of breast cancer cells. *The mimic of miR-320a was transfected in MCF-7 cells and clonogenic assay was performed in untreated (A) and TNF-α treatments (B). The total cellular RNA was prepared from MCF-7 cells transfected with mimic. The relative expression of the genes regulating glycolysis was analyzed by real time PCR (C). To further elucidate the role of ROS in tumorigenesis, MCF-7 was transfected with control and miR-320amimic and treated with NAC (D). The expression pattern of miR-320a in tumor tissue was determined qPCR (E) (n=5).* $^{*}P < 0.01$, $^{***}P < 0.0001$.

To further confirm we monitored its level in tumor and extra-tumoral tissue of breast cancer. As expected the level of miR-320a was high in extra tumoral tissue as compared to normal tissue (Figure 8.8E). In conclusion miR-320a regulates OXPHOS, glycolysis and ROS balance and hence tumorous tissues loose its expression for metabolic reprogramming.

8.6 Discussion

The mitochondria essentially depend on nuclear genome encoded RNA and proteins for its specific and optimal functioning. The stalling of mitochondria and enrichment of associated RNAs at particular sub-cellular location (Lin and Sheng, 2015) is observed in

neurons. The mitochondria are asymmetrically distributed towards migration axis of invasive cancer cells (Desai et al., 2013) suggesting the role of mitochondria in subcellular site specific protein synthesis. However, the association of miRNAs to mitochondria and its functional implication in regulation of mRNA level is not understood. In the current study, we demonstrate the association of miR-320a at outer membrane of mitochondria, its role in regulation of activity of mitochondrial complex-I and its implication in tumorigenesis.

The transport of nuclear encoded miRNAs to mitochondria is now emerging as one of the major mechanisms of fine regulating the mitochondrial functions as well as provides tissue specific function. This process is not well understood. The previous report from our group and others suggest the possibility of two populations of nuclear encoded miRNAs: one that may be present on outer mitochondrial membrane or in close vicinity. The other group of miRNAs translocates to mitochondrial matrix and may bind either to mitochondrial DNA or encoded transcripts and regulates its cognate mitochondrial subunit. This hypothesis has been supported by the recent reports where miR-181c and miR-151a-5p have been found in mitochondria and where they regulate the level of COX1 and CYTB respectively (Das et al., 2014; Zhou et al., 2015). Similarly, miR-1 has been implicated in muscle myogenesis by modulating the levels of ND1 and COX1 inside the mitochondria (Zhang et al., 2014). In our recent report, we have observed that miR-4485 translocates to mitochondria and regulates 16S rRNA pre-mRNA processing of mitochondrial genome encoded transcripts. The second group comprises of pre-miRNA and mature miRNAs that are found in close vicinity to mitochondria. Emerging evidences suggest that mitochondria is not isolated entity in cell as and its cross talk with Endoplasmic Reticulum (Filadi et al., 2017), nucleus (Cagin and Enriquez, 2015), peroxisome (Demarquoy and Le Borgne, 2015), Stress granules (Souquere et al., 2009), melanosomes (Wu and Hammer, 2014), purinosomes (French et al., 2016), P-bodies (Huang et al., 2011) and ribosome (Gehrke et al., 2015) is emerging. In this study we observed that miR-320a in normal physiological conditions is present in cytosol whereas it is enriched in mitochondrial fraction in the presence of TNF-α. The association to mitochondria is altered in different stressed conditions. Moreover experiments here suggest that miR-320a is associated with specific mitochondrial targeted RNA binding protein. The enrichment of miR-320a both in CLUH

and Ago-2 may have different implication as observed here however it needs to be further studied. Recently, the transport of nascent mRNA of nuclear encoded mitochondrial protein to mitochondrial outer membrane was observed. The transport is regulated by RNA binding proteins like CLUH (Gao et al., 2014). Interestingly the level of NDUFS1 (catalytic subunit in matrix) increased whereas NDUFA10 (accessory subunit at inner membrane) decreased on mitochondrial surface. This is interesting as both mRNA are subunit of complex-I and targets of miR-320a however their association to mitochondria through different RNA binding proteins. NDUFA10 binds to Ago-2 hence it may be degraded through Ago-2 mediated silencing however NDUFS1 mRNA preferentially enriches to mitochondria via CLUH hence it may not be regulated Ago-2 mediated silencing hence it its level remained high. This suggests that nuclear encoded mRNA binds to RNA binding protein like CLUH and is transported to mitochondria where the specific miRNA enriches in stimulus specific conditions to regulate the copy number. This hypothesis is further supported by recent observation of cytosolic ribosome at outer mitochondrial membrane. The localized protein synthesis of nmRNAmp at mitochondrial surface was demonstrated. The process involves PINK1 and TOM mediated recruitment of nascent nmRNAmp and ribosomes. These evidences strongly support our novel hypothesis of miRNA mediated fine tuning the nuclear encoded mRNA of mitochondrial targeted protein. MiR-320a regulates the level one such nmRNAmp, NDUFA10 on the outer membrane of mitochondria. MiR-320a also modulates the function of mitochondrial Complex-I activity by modulating the mitochondrial super complex-I assembly. The mitochondrial Complex-I comprises of 45 subunits, 37 of which are nuclear encoded. Here we observed that NDUFA10 and NDUFS1 are putative targets of miR-320a. N-terminal region of NDUFA10 is hydrophobic and anchored at inner membrane, while its C-terminal is hydrophilic and turns towards matrix, conferring L-shaped structure to the complex (Hoefs et al., 2011). The downregulation of NDUFA10 may not allow the assembly of the super complex hence we observed the sub complex-I in miR-320a transfected cells and overall mitochondrial defects. The level of NDUFS1, a subunit of N-module of complex-I, localized at the tip of hydrophilic arm towards matrix is target and sequester on mitochondrial outer membrane predominantly through CLUH, hence it may not be accessible for the degradation through miRISC complex. This may be novel strategy of

mitochondria to prevent the degradation of critical nuclear encoded nmRNAmp. This requires further experimentation and proof in different pathophysiological conditions.

The reprogramming of mitochondrial function is one of the metabolic adaptations of the cancer cells (Vyas et al., 2016). The inhibition of mitochondrial functions suggests its role as probable tumor suppressor. The observations here clearly support miR-320a as potential tumor suppressor as miR-320a inhibits clonogenic and migration ability of the cancer cells. Our hypothesis is supported by many previous reports showing that miR-320a may act as potential tumor suppressor in cancer of different origins (Lv et al., 2017; Ma et al., 2017; Tadano et al., 2016; Wang et al., 2016; Yu et al., 2016; Zhu et al., 2016). MiR-320a is encoded from chromosome 8, which is lost in many tumors (Knösel et al., 2004; Macartney-Coxson et al., 2008) further supporting our hypothesis. In consonance to this observation we further observed that miR-320a expression is decreased in tumorous region of the breast cancer. The increased level of miR-320a is known to inhibit glycolysis (Tang et al., 2012). The report here suggest that loss of miR-320a during tumorigenesis provide dual advantage, increased OXPHOS capacity for increased level of precursors for macromolecular synthesis and increased level of glycolysis for energy for rapid proliferation.

In conclusion, this study suggests the existence of unique population of nuclear genome transcribed miRNA which selectively enriches on mitochondria in the presence of TNF-α. This study further suggests the different pattern of miRNAs enrichment to mitochondria in other patho-physiological stimuli suggesting the dynamic association of miRNAs to the outer membrane of mitochondria. This study demonstrated unique mechanism of regulation of the nuclear encoded mRNA levels of mitochondria targeted protein through Ago-2 mediated silencing or translation and protection via CLUH. The nuclear encoded miRNAs and transport to mitochondria in specific physiological conditions is another mode of control and providing specificity to mitochondrial function. These possibilities further needs to be investigated.