Chapter Five:

NLRX1 acts as a tumor suppressor by modulating TNF-α-regulated mitochondrial function and apoptosis in cancer cells

Chronic inflammation is closely associated with tumor initiation, promotion and progression. TNF-α is a multifunctional cytokine that mediates chronic inflammatory condition and promotes tumor cell survival. Increased level of TNF-α has been consistently observed in different tumor types (Lippitz, 2013). The role of mitochondria in regulation of inflammatory pathways is emerging. NLRX1, a mitochondrial NOD-like receptor protein, negatively regulates innate immune signaling pathways to limit acute inflammatory response during infection (Allen, 2014). NLRX1 expression is differentially regulated in non-immune cells including cancer cells, however its role in regulation of cell death and metabolism and its implication in tumorigenesis are not well understood. Here, we investigated the role of NLRX1 in regulating mitochondrial metabolism and its effect in modulation of TNF-α induced apoptosis in cancer cells.

5.1 NLRX1 sensitizes TNF-α -induced cell death by activating caspase-8.

To understand the role of NLRX1 in regulation of cell death and survival in response to different physiological stress, we transfected HEK293 cells with NLRX1 and monitored cell death in the presence of different stimuli by trypan blue exclusion assay (Fig. 5.1A). The over-expression of NLRX1 showed no effect on cell survival in untreated conditions. Similarly, the oxidative stress-induced cell death in presence of rotenone and H_2O_2 treatment did not vary significantly between control and NLRX1- transfected cells. In contrast, we observed a significant increase in cell death in NLRX1-transfected cells as compared to control in the presence of TNF-α and cycloheximide (CHX). A similar increase in cell death was observed in NLRX1-transfected HeLa cells in the presence of TNF-α/CHX as compared to vector-transfected cells (Fig. 5.1B). These results indicated that ectopic expression of NLRX1 specifically sensitizes TNF-α-induced cells death. To confirm this, we depleted endogenous NLRX1 using NLRX1-specific shRNAs in HEK293cells. Three different shRNAs were checked for the efficient knockdown of NLRX1.We found thatshRNA1 of NLRX1 was most potent and hence we further used it in the study (Fig. 5.1C). A significant decrease in cell death was observed in NLRX1 knockdown (NLRX1-KD) cells as compared to control in the presence of TNF-α/CHX (Fig. 5.1D). Similarly, knockdown of NLRX1 increased cell survival in the presence of

TNF-α/CHX when cell viability was assessed using MTT assay (Fig. 5.1E). These results confirmed that NLRX1 is an important regulator of TNF-α induced cell death.

To further investigate the mechanism of TNF-α-induced cell death, we monitored PARP cleavage in NLRX1-KD cells in the presence of TNF-α/CHX by immunoblotting. PARP is cleaved by executioner caspases during TNF-α-induced apoptosis. The western blotting showed 110 kDa and 89 kDa band corresponding to native and cleaved form of PARP in control shRNA transfected cells. The knockdown of NLRX1 showed significant decreased levels of 89 kDa bands corresponding to cleaved subunit of PARP as compared to control (Fig. 5.1F, G). The cleavage of PARP suggest the activation of caspases in presence of NLRX1. To rule out the cell line specific role, ectopic expression of NLRX1 in MCF-7 breast cancer cells also showed increased levels of cleaved PARP subunit as compared to vector transfected cells. The cleavage of PARP during TNF-α-induced cell death suggested the activation of caspases in NLRX1-transfected cells (Fig. 5.1H). The activation of caspases play an important role in induction and execution of TNF-α-mediated apoptosis (Micheau and Tschopp, 2003) hence we investigated the role of caspases in the NLRX1-mediated sensitization to the TNF-α-induced cell death. We analyzed the activation of caspase-8 and caspase-3 activation at different time points by immunoblotting (Fig. 5.1I). An increased level of 41 kDa and 18 kDa bands corresponding to cleaved subunits p43 and p18 of caspase-8 and a 17 kDa band corresponding to the cleaved subunit of caspase-3 were detected as early as 4 h in NLRX1-transfected HEK293 cells as compared to control in the presence of TNF-α/CHX. The quantification of caspase-8 proteolytic activity showed a similar increase in caspase-8 activation at 4 h which remained elevated till 8 h in NLRX1-transfected cells in the presence of TNF-α/CHX (Fig. 5.1J). Conversely, analysis of caspase-8 activation by immunoblotting in NLRX1-KD cells revealed a significant decrease in the levels of cleaved caspase-8 subunits (p43 and p18) in the presence of TNF-α/CHX as compared to control (Fig. 5.1K). Similarly, an increase levels of cleaved caspase-8 subunits were detected in NLRX1-expressing MCF-7 cells as compared to vector transfected cells in the presence of TNF-α/CHX (Fig. 5.1H). Altogether, these data suggested that NLRX1 sensitizes TNF-α-induced cell death by promoting the early activation of caspase-8.

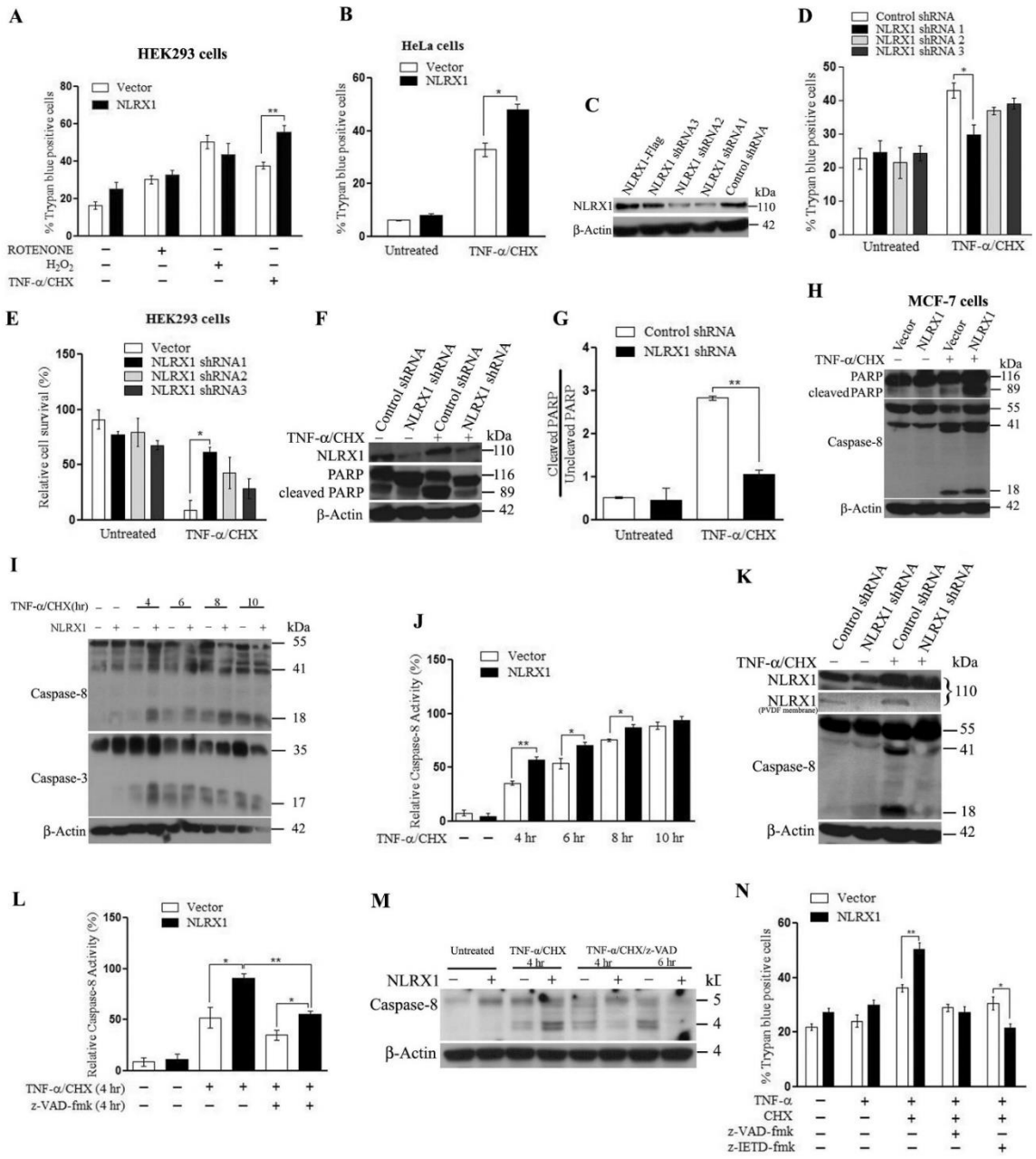

Figure 5.1: NLRX1 regulates TNF-α -induced cell death by promoting caspase-8 activation. (A) *and* ***(B)*** *HEK293 and HeLa cells were transfected with NLRX1 and vector control followed by treatment with Rotenone (50 μM), H2O2 (100 μM) or TNF-α/CHX (10 ng/25 μM). The cell death was monitored by Trypan blue exclusion assay.* ***(C)*** *and* ***(D)*** *HEK293 cells were transfected with control shRNA, NLRX1-Flag and three different shRNAs targeting NLRX1, namely NLRX1 shRNA 1, 2 and 3. Cell lysates were analyzed for NLRX1 expression by western blotting. After confirmation of knockdown, HEK293 cells were transfected with control shRNA and three shRNA targeting NLRX1. Cells were subjected to TNF-α/CHX treatment for 24 h and analyzed cell death by Trypan blue staining.****(E)*** *HEK293 cells were transfected with three different shRNAs targeting NLRX1. After 36 hours of transfection, cells were treated with TNF-α/CHX for 24*

hours and cell proliferation was measured using MTT assay. ***(F)*** *and* ***(G)*** *NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α/CHX for 6 h. Cell lysates were subjected to western blotting with PARP specific antibody. Quantification of PARP cleavage was performed using densitometric analysis of bands and ratios of cleaved to uncleaved PARP are indicated.* ***(H)*** *MCF-7 cells were transfected with NLRX1 and vector followed by treatment with TNF-α/CHX for 4 hours. Cell lysates were analyzed by western blotting with PARP and Caspase-8 specific antibody.* ***(I)*** *HEK293 cells were transfected with NLRX1 and treated with TNF-α/CHX for indicated time. The cell lysates were analyzed by western blotting using caspase-8 and caspase-3 specific antibodies.****(J)*** *HEK293 cells were transfected and treated as indicated in (I) and caspase-8 activity was analyzed at different time points by caspase-8 Glo luciferase assay system (n = 2).* ***(K)*** *NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α/CHX for 4 h and caspase-8 activation was analyzed by western blotting.* ***(L)*** *and* ***(M)*** *HEK293 cells were transfected with NLRX1 and vector and treated with TNF-α/CHX or TNF-α/CHX and z-VAD-fmk (20 μM) for indicated time. The caspase-8 activation was analyzed by Caspase-8 Glo luciferase assay system (n = 2) (L) and western blotting (M).* ***(N)*** *HEK293 cells were transfected with NLRX1 and vector control followed by treatment with TNF-α/CHX, z-VAD-fmk (20 μM) and z-IETD-fmk (10 μM). After 24 h of treatment, cell death was measured by trypan blue exclusion assay. Data in (A), (B), (D), (E) and (N) depict mean ± SEM values (n = 3). Asterisk (*) indicates that p value < 0.05, for SEM.*

To confirm this, we further quantified caspase-8 activity in the presence of TNF-α/CHX with or without z-VAD-fmk, a pan-caspase inhibitor (Fig. 5.1L). A decrease in caspase-8 activity was observed following inhibition of caspases, which declined significantly in NLRX1-transfected cells as compared to control. Similarly, the analysis of caspase-8 activation by immunoblotting showed reduced levels of the cleaved caspsase-8 subunits at 4 h which decreased further by 6 h in NLRX1-transfected cells co-treated with z-VAD-fmk (Fig. 5.1M). Finally, we monitored cell death in NLRX1-transfected cells in the presence of TNF-α either alone or co-treated with CHX, z-VAD-fmk and z-IETD-fmk, a caspase-8 specific inhibitor, by trypan blue exclusion assay (Fig. 5.1N). While the cell death was equally reduced in both control and NLRX1-transfected cells in the presence of TNF-α/CHX and z-VAD-fmk, co-treatment with z-IETD-fmk significantly increased cell survival in NLRX1-tranfected cells. Collectively, these evidences strongly suggested the role of NLRX1 in regulation of caspase-8 activation during TNF-α-induced cell death.

5.2 NLRX1 promotes caspase-8 activation by associating with TNF-α-induced complex-II.

The formation of complex-II upon ligation of TNF-α to its cell surface receptor initiates cell death program by recruiting caspase-8 and other adaptor proteins. An increased activation of caspase-8 activity by NLRX1 as early as 4 h after TNF-α/CHX treatment suggested that NLRX1 may act at the level of complex-II formation. Previous studies indicated an essential role of ubiquitin ligases in the assembly and activation of casaspe-8. RING family E3 ligase TRAF2 interacts with caspase-8 at complex-II and sets a threshold for its activation (Gonzalvez et al., 2012). Similarly, we demonstrated the role of the RING E3 Ligase, TRIM13 in regulation of caspase-8 activation downstream of complex-II (Tomar et al., 2013). Therefore, we planned to investigate the role of the E3 Ligases in the regulation of the NLRX1 mediated activation of caspase-8 and its association with TNF-α-induced signaling complexes. Analysis of caspase-8 activation by immunoblotting revealed an increased level of 41 kDa and 18 kDa bands corresponding to processed caspase-8 in TRIM13 transfected cells in the presence of TNF-α/CHX (Fig. 5.2A). This result is in agreement with our previous observation (Tomar et al., 2013). Similarly, in agreement with earlier report, ectopic expression of TRAF2 attenuated the processing and activation of caspase-8 to that of control. Interestingly, co-expression of either TRIM13 or TRAF2 with NLRX1 decreased the activation of caspase-8 in presence of TNF-α/CHX. Taken together, these results supported our hypothesis that NLRX1 regulates caspase-8 activity at the level of complex-II formation after TNF-α stimulation.

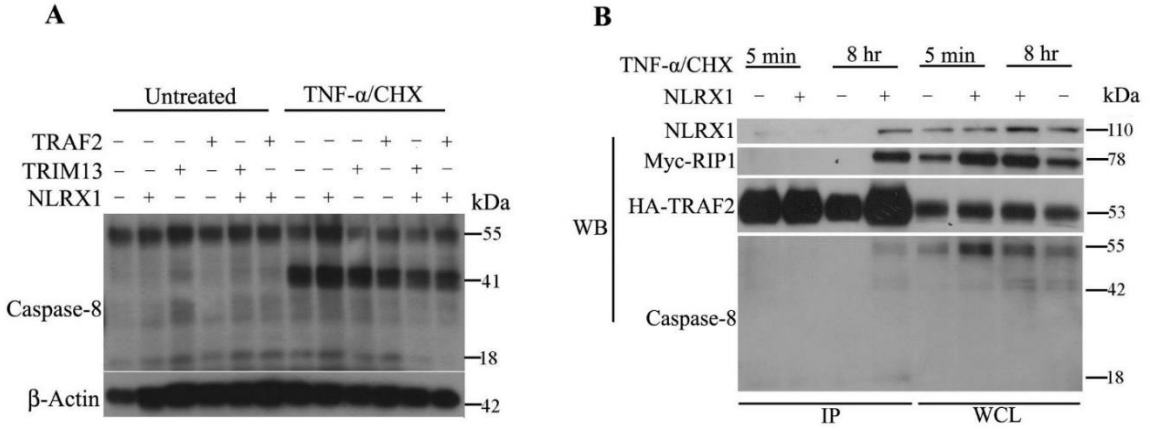

Figure 5.2: NLRX1 increases caspase-8 activation by interacting with TNF-α-induced complex-II. (A) *HEK293 cells were cotransfected with NLRX1 in combination with TRAF2 and TRIM13. The cells were treated with TNF-α/CHX for 4 h. Cell lysates were analyzed by Western blotting with caspase-8 antibody.* ***(B)*** *HEK293 cells were transfected with NLRX1-Flag, HA-TRAF2, Myc-RIP1 followed by co-treatment with TNF-α/CHX for 5 min and 8 h. Immunoprecipitation with anti-HA antibody was performed and interacting proteins were analyzed by western blotting with indicated antibodies.*

To test whether NLRX1 may associate with the components of complex-II to regulate caspase-8 activity, we performed co-immunoprecipitation (IP) analysis. To detect the formation of TNF-α-induced complex-I or complex-II, we co-expressed NLRX1 along with HA-TRAF2 and Myc-RIP1 in HEK293 cells, and immunoprecipitated TRAF2, a subunit of both complexes with anti-HA antibody following treatment with TNF-α/CHX for 5 min or for 8 h (Fig. 5.2B). The analysis of interaction by immunoblotting identified a 110 kDa band corresponding to NLRX1, a 78 kDa band of RIP1 and casaspe-8, after eight hours of TNF-α /CHX treatment. We did not detect the pull down of either NLRX1, RIP1 or caspase-8, after five mins of TNF-α/CHX treatment. These results indicated that NLRX1 associates with TNF-α-induced signaling complex-II to promote the activation of caspase-8.

5.3 NLRX1 localizes to mitochondria and regulates TNF-α-induced ROS generation.

Previous studies suggested that NLRX1 is a mitochondrial-resident protein however, its functional significance was not known. Similarly, the active pool of caspase-8 has been reported to localize to mitochondria (Gonzalvez et al., 2008), hence, we hypothesized that the crosstalk between NLRX1 and casaspe-8 may regulate mitochondrial functions in response to TNF-α. We analyzed the sub-cellular localization of NLRX1 using a c-terminal GFP fusion construct, NLRX1-GFP, transfected into MCF-7-mtRFP stable cell line by confocal microscopy (Fig. 5.3A). We observed a significant fraction of NLRX1 co-localizing with mitochondria in the presence of TNF-α (Fig. 5.3B). This observation was further confirmed by sub-cellular fractionation and immunoblotting. We observed a specific enrichment of NLRX1 and pro-caspase-8 in the mitochondrial fraction of both untreated and treated cells (Fig. 5.3C). Interestingly, an increased levels 43 kDa and 18 kDa bands corresponding to the cleaved subunit of Caspase-8 (p43 and p18) was detected mi-

tochondrial fraction of NLRX1-transfected cells as compared to vector control in the presence of TNF-α/CHX. The quality of mitochondrial and cytosolic fractions was further analyzed using antibodies to NDUFS2, a subunit of mitochondrial Complex I, and to RPS9, a component of 40S ribosomal subunit respectively. NDUFS2 was found exclusively in the mitochondrial fraction whereas RPS9 was detected only in the cytosol confirming the purity of subcellular functions. Together, these results demonstrated that NLRX1 localizes to mitochondria in the presence of TNF-α/CHX.

Emerging evidences suggest an important role of caspases in mediating the proteolytic degradation of key subunits of OxPhos complex to regulate ROS generation during different stress conditions (Huai et al., 2013). The role of crosstalk between NLRX1 and caspase-8 in regulation of mitochondrial respiratory function and ROS generation is not well understood. To test whether NLRX1 may regulate TNF-α-induced ROS generation, we monitored the levels of intracellular ROS in NLRX1-KD HeLa cells in the presence of TNF-α/CHX by staining cells with an oxidant-sensitive dye, CM-H_2DCFDA (Fig. 5.3D). As expected, the ROS levels in control cells increased in the presence of TNF-α/CHX. The knockdown of NLRX1 significantly decreased ROS generation in the presence of TNF-α/CHX. To confirm this, we further quantified intracellular ROS levels in NLRX1-KD HEK293 cells in the presence of N-Acetyl Cysteine (NAC, an antioxidant) and TNF-α/CHX (Fig. 5.3E). ROS generation was significantly decreased in NLRX1-KD cells while co-treatment with NAC further suppressed ROS formation in the presence of TNF-α/CHX. Based on these evidences, we concluded that NLRX1 localizes to mitochondria and regulates TNF-α-induced ROS generation.

Mitochondria is one of the major source of ROS release during TNF-α-induced cell death. We hypothesized that a crosstalk between NLRX1 and caspase-8 may regulate mitochondrial functions, including the generation of ROS. To test this possibility, we monitored the levels of mitochondrial superoxide generation in NLRX1-transfected HEK293 cells in the presence of TNF-α/CHX. The ectopic expression of NLRX1 alone stimulated mitochondrial superoxide formation in untreated condition which further increased significantly in the presence of TNF-α/CHX as compared to vector control (Fig. 5.3F). Similarly, we further quantified mitochondrial superoxide generation in these cells in the

presence of Mito-TEMPO, a mitochondrial superoxide scavenger and TNF-α/CHX (Fig. 5.3G). NLRX1 expression significantly upregulated mitochondrial ROS generation in the presence of TNF-α/CHX while co-treatment with Mito-TEMPO suppressed superoxide formation as compared to vector control.

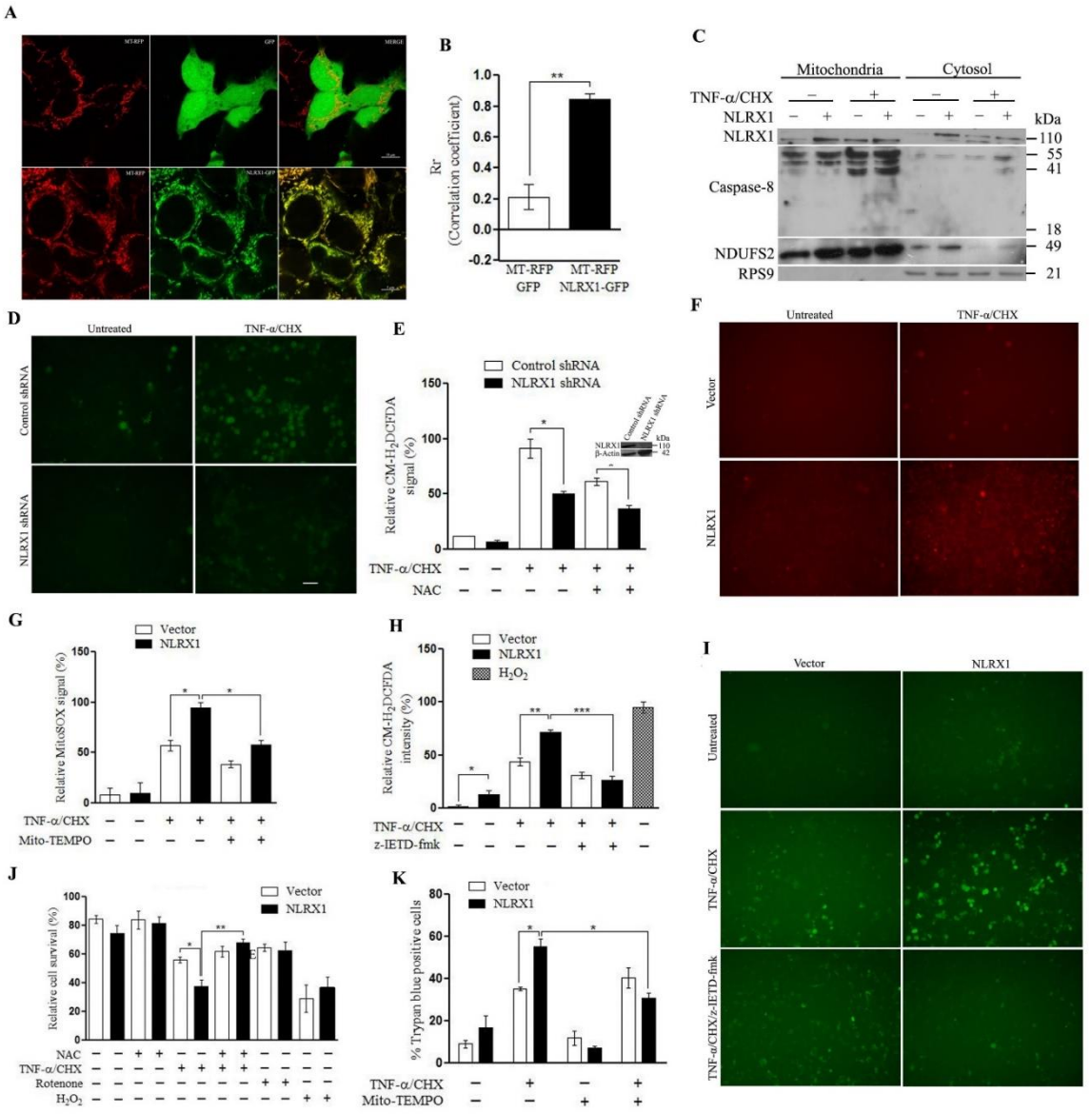

Figure 5.3: NLRX1 localizes to mitochondria and regulates mitochondrial ROS generation during TNF-α-induced caspase-8 activation. (A) *MCF-7-mt-RFP stable cell line was transfected with NLRX1-GFP and GFP control. After 24 h of transfection, cells were treated with TNF-α, fixed with 4% paraformaldehyde and visualized by confocal microscopy. Scale bar in the upper and lower panel represents 10 μm and 5 μm, respectively.* ***(B)*** *Colocalization quantification of confocal images by Pearson's correlation coefficient (Rr) was performed using Image J 1.45 software (NIH, MD, USA).* ***(C)*** *HEK293 cells were transfected with*

NLRX1 and control vector followed by treatment with TNF-α/CHX for 4 h. Cells were subjected to subcellular fractionation. Mitochondrial and cytoplasmic fractions from untreated and treated cells were analyzed by western blotting with indicated antibodies. ***(D)*** *NLRX1 shRNA and control shRNA stable HeLa cells were treated with TNF-α/CHX for 4 h. ROS production was monitored by staining the cells with CM-H_2DCFDA fluorescent probe and visualized under fluorescence microscope. Scale bar represents 10 μm.* ***(E)*** *Quantification of ROS level: control shRNA and NLRX1 shRNA stable HEK293 cells were treated with TNF-α/CHX in the absence/presence of NAC (5 mM) using CM-H_2DCFDA.* ***(F)*** *HEK293 cells were transfected with NLRX1 and vector control and treated with TNF-α/CHX for 4 hours. Mitochondrial superoxide levels were analyzed by staining cells with MitoSOX Red and visualized using fluorescence microscopy.* ***(G)*** *Quantification of mitochondrial superoxide levels in NLRX1 and vector transfected MCF-7 cells treated with TNF-α/CHX in the absence/presence of Mito-TEMPO (10 μM).* ***(H)*** *Intracellular ROS levels were quantified in NLRX1 and vector transfected MCF-7 cells following treatment with TNF-α/CHX and z-IETD-fmk either alone or in combination. H_2O_2 treatment (50 μM) was used as a positive control.* ***(I)*** *HeLa cells were transfected and treated as indicated in (H) and the cells were stained with CM-H_2DCFDA and ROS levels were monitored under fluorescence microscope.* ***(J)*** *MCF-7 cells were transfected with NLRX1 and vector control followed by treatment with NAC (5mM), TNF-α/CHX, Rotenone and H_2O_2 for 24 hours and cell proliferation was measured using MTT assay.* ***(K)*** *NLRX1 and vector transfected HEK293 cells were treated with TNF-α/CHX and Mito-TEMPO either alone or in combination and cell death was monitored by Trypan blue exclusion assay. Data in (E), (G), (H), (J) and (K) depict mean ± SEM values (n = 3). Asterisk (*) indicates that p value < 0.05, for SEM.*

To determine the role of caspase-8 activation in regulation of TNF-α induced ROS generation, we monitored intracellular ROS levels in NLRX1-transfected HeLa cells in the presence of TNF-α/CHX pretreated with z-IETD-fmk (Fig. 5.3I). The ROS levels were significantly increased in NLRX1 transfected cells in the presence of TNF-α/CHX as compared to vector control. The inhibition of caspase-8 activity with z-IETD-fmk suppressed ROS generation in NRX1-transfected cells treated with TNF-α/CHX. Similarly, quantification of ROS levels in MCF-7 cells transfected with NLRX1 showed similar results both in the presence of TNF-α/CHX and co-treatment with z-IETD-fmk (Fig. 5.3H). We also tested whether blocking ROS generation may rescue TNF-α-induced cell death in NLRX1 expressing MCF-7 and HEK293 cells. As observed earlier, ectopic expression of NLRX1 did not affected cell survival in the untreated conditions and in the presence of Rotenone and H_2O_2 but significantly increased cell death upon TNF-α/CHX stimulation (Fig. 5.3J). In contrast, co-treatment with NAC significantly increased cell survival of

NLRX1-transfected cells as compared to control. Similar results were observed in NLRX1-transfecetd HEK293 cells in the presence of TNF-α/CHX and Mito-TEMPO (Fig. 5.3K). All together, these data suggested that NLRX1 regulates caspase-8 activity to modulate mitochondrial ROS generation and cell death during TNF-α induced apoptosis.

5.4 NLRX1 alters cellular ATP levels by modulating TNF-α-regulated mitochondrial function.

Mitochondrial complex I and complex III of OxPhos system are the major sites of ROS generation (Hamanaka and Chandel, 2010), hence we examined if ectopic expression of NLRX1 may regulate their activity. We observed a significant decrease in enzyme activity of complex I in NLRX1-transfected HEK293 cells both in absence and presence of TNF-α as compared to control (Fig. 5.4A). Co-treatment with TNF-α/CHX equally reduced the complex I activity in both cells. Interestingly, inhibition of caspase-8 specifically by z-IETD-fmk but not with z-VAD-fmk significantly increased complex I activity in the presence of TNF-α/CHX. Conversely, the knockdown of NLRX1 in HEK293 cells increased both complex I and complex III activity in absence and presence of TNF-α as compared to control cells (Fig. 5.4B, C, D) The above results suggested that NLRX1 regulates the activity of mitochondrial respiratory chain complexes which is the major source of intracellular ATP. Therefore, we further monitored the levels of both total and mitochondrial ATP in the presence of TNF-α.The ectopic expression of NLRX1 in HEK293 cells decreased total cellular steady-state ATP level in the presence of TNF-α as compared to control whereas this decrease was less evident when the cells were co-treated with TNF-α and CHX (Fig. 5.4E). Similar results were observed in MCF-7 cells where ectopic expression of NLRX1showed a significant decrease in total ATP levels both in the absence and presence of TNF-α (Fig. 5.4G). The ATP levels were measured after 16 h of treatment with TNF-α/CHX, hence, the activated caspases may have cleaved the subunits of OxPhos complexes as observed earlier and hence the decrease may not be evident. Importantly, the inhibition of caspase-8 by z-IETD-fmk during TNF-α/CHX-induced apoptosis significantly enhanced the ATP levels in NLRX1-transfected cells as compared to control (Fig. 5.4E). Co-treatment with z-VAD-fmk showed no significant

effect which is possible as z-VAD-fmk is not a potent inhibitor of the initiator caspases further suggesting that activated caspase-8 may directly act on OxPhos complexes under ectopic expression of NLRX1. The knockdown of NLRX1 in HEK293 cells significantly increased intracellular ATP levels both in the presence of TNF-α and TNF-α/CHX as compared to control (Fig. 5.4F). The mitochondrial ATP levels in NLRX1-KD cells increased both in the absence and presence of TNF-α (Fig. 5.4H). These results strongly suggested that NLRX1participates in regulation of mitochondria complexes activity and play an important role in the maintenance of intracellular ATP levels.

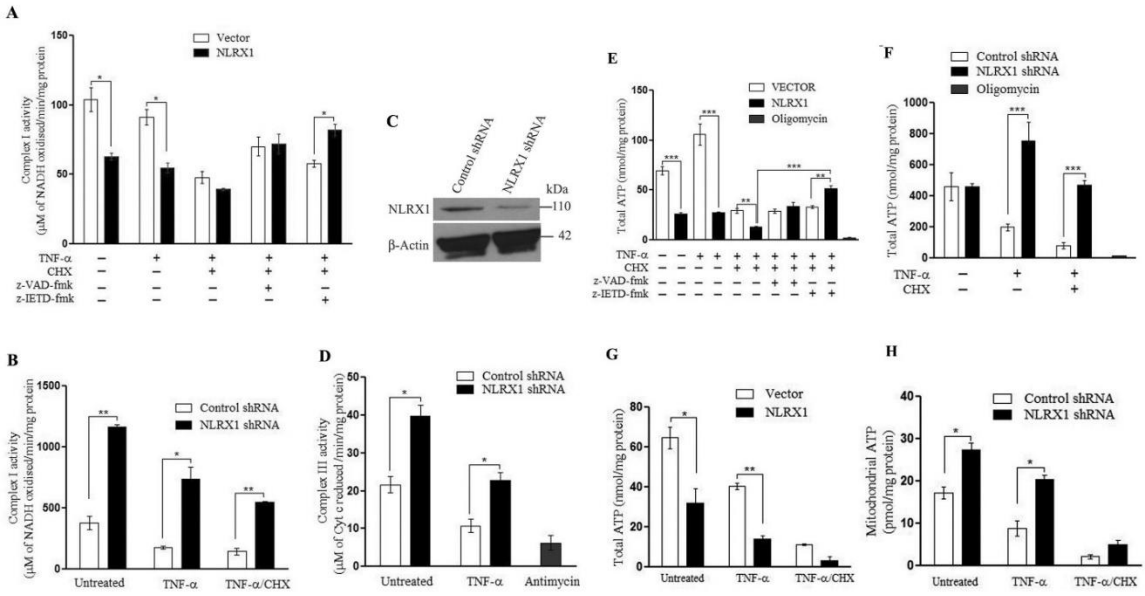

Figure 5.4: NLRX1 regulates ATP levels by modulating mitochondrial complex I and complex III activity. (A) *HEK293 cells were transfected with NLRX1 and vector control followed by treatment with TNF-α, TNF-α/CHX, z-VAD-fmk and z-IETD-fmk either alone or in combination.* ***(B)*** *and* ***(D)*** *NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α and TNF-α/CHX. Complex I and Complex III activity were measured spectrophotometrically.* ***(C)*** *Cell lysates of NLRX1 shRNA stable HeLa cells were analyzed by western blotting to check NLRX1 expression levels.* ***(E)*** *HEK293 cells were transfected with NLRX1 and vector control followed by treatment with TNF-α, TNF-α/CHX, z-VAD-fmk and z-IETD-fmk either alone or in combination.* ***(F)*** *NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α and CHX either alone or in combination. ATP levels in (E) and (F) were measured by ATP-dependent luciferase activity.* ***(G)*** *NLRX1 shRNA1 and control shRNA stable HEK293 cells were treated with either TNF-α alone or in combination with CHX. Mitochondria were isolated.* ***(H)*** *MCF-7 cells were transfected and treated as indicated in (E). ATP levels in (G) and (H) were measured as in (E). Data depicts mean ± SEM (n=3). Asterisk (*) indicates that p value < 0.05, for SEM.*

5.5 NLRX1 suppresses tumorigenic potential of cancer cells both *in vitro* and *in vivo*.

NLRX1-regulated decrease in mitochondrial respiratory function suggested its important role in modulation of cancer cell metabolism and hence may regulate clonogenic ability and tumorigenic potential of cancer cells. NLRX1 expression levels across different tumor types available in gene expression databases, reported by earlier studies, suggested that NLRX1 is significantly downregulated in primary breast tumors, cervical, colon and hepatocellular carcinoma (Coutermarsh-Ott et al., 2016; Hu et al., 2018; Lei and Maloy, 2016). Therefore, we examined NLRX1 expression pattern in different breast cancer cell lines using immunoblotting. The protein levels of NLRX1 were low in MCF-7 and T47D (ER and PgR positive) cells as compared to MDA-MB-231 and HBL-100 (ER and PR negative) cells which showed an elevated level of NLRX1 (Fig. 5.5A). We investigated if ectopic expression of NLRX1 in MCF-7 affected cancer phenotype using clonogenic assay and soft agar tumorigenesis assay.The clonogenic ability of NLRX1 expressing MCF-7 cells was monitored in the presence and absence of TNF-α (Fig. 5.5B). NLRX1 expression significantly decreased the clonogenic ability of MCF-7 cells (shown as plating efficiency) in presence of TNF-α (Fig. 5.5B'). The soft agar assay was performed to study anchorage-independent cell growth.The control cells showed normal growth in soft agar and formed large colonies (plotted as colony area,) in the presence of TNF-α (Fig. 5.5C and C') while NLRX1-transfected cells were unable to grow in soft agar and formed small colonies in the of TNF-α, as compared to untreated cells.We further analyzed the migration ability of these cells in the presence of TNF-α by scratch assay (Fig. 5.5D). We observed a significant increase in open wound area after 24 h in NLRX1-transfected as compared to control in the presence of TNF-α. The low levels of NLRX1 expression in MCF-7 cells may regulate normal cellular functions hence we depleted endogenous NLRX1 using shRNA and monitored its growth in soft agar.The knockdown of NLRX1 increased the ability of MCF-7 cells to form colonies (tumorispheres) in soft agar as evident by the increased colony size in the presence of TNF-α in comparison to control (Fig. 5.5E, F and G). Collectively, these results suggest that NLRX1 negatively regulates tumorigenic potential and migration ability of breast cancer cells both in absence and presence of TNF-α.

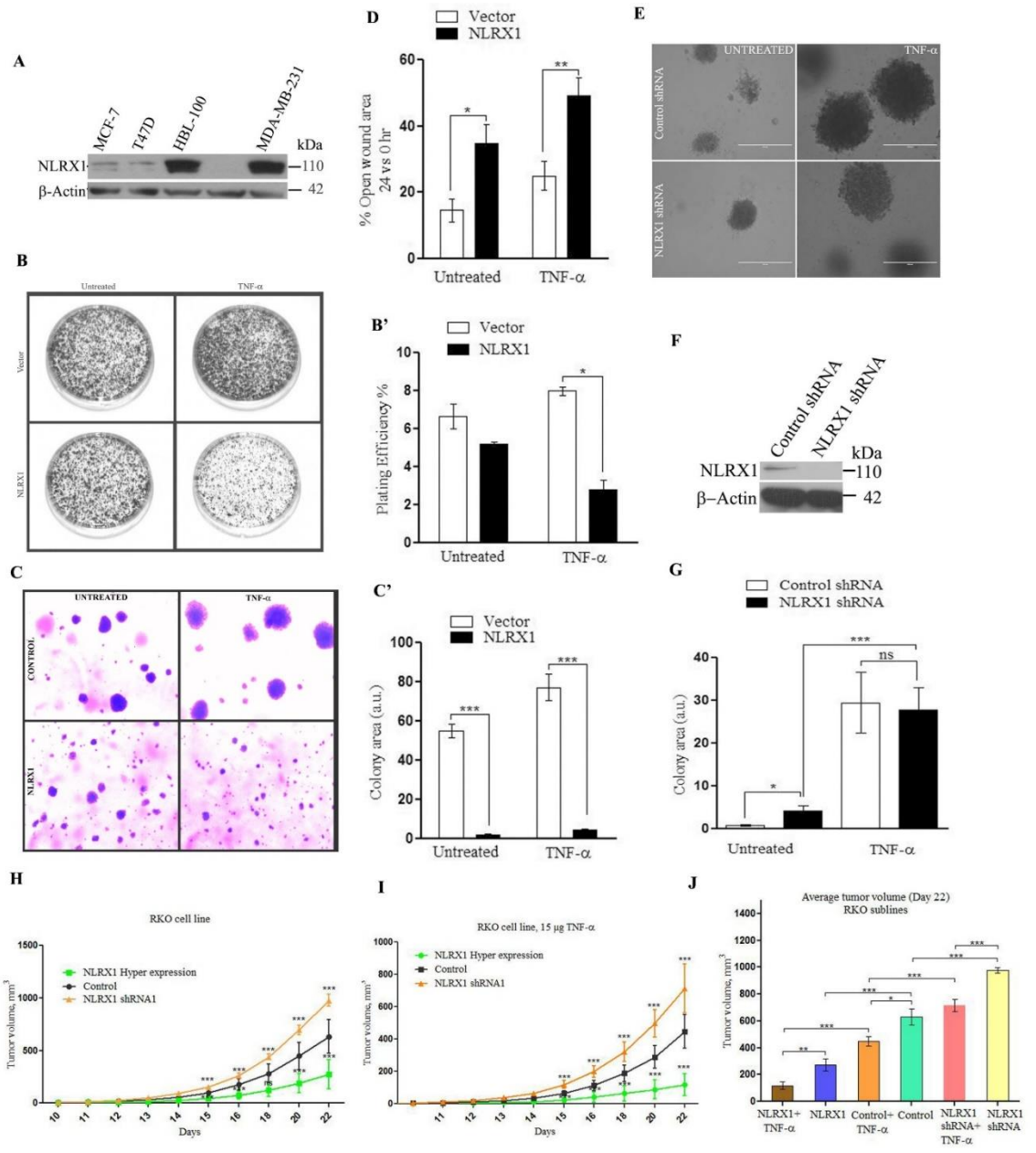

Figure 5.5: NLRX1 expression regulates the tumorigenicity of cancer cells both in vitro and in vivo. (A) *The expression of NLRX1 was analyzed by western blotting in MCF-7, T47D, HBL-100 and MDA-MB-231.* ***(B)*** *and* ***(B')*** *MCF-7 cells were transfected with NLRX1 and control vector followed by treatment with TNF-α and clonogenic activity was assessed by counting number of colony forming units and plotted as plating efficiency.* ***(C)*** *and* ***(C')*** *MCF-7 cells were transfected with NLRX1 and vector constructs and treated with TNF-α. The soft agar assay was performed and colonies in soft agar were observed under the microscope after 25 days. The colonies areas were measured using Image J 1.45 software.* ***(D)*** *MCF-7 cells transfected with NLRX1 and control vector were subjected to TNF-α treatment and scratch assay was performed as indicated in the Materials and methods section.* ***(E)*** *and* ***(G)*** *NLRX1 shRNA1 and control shRNA*

transfected MCF-7 cells were treated as indicated in (C) and soft agar assay was performed. ***(F)*** *NLRX1 knockdown was confirmed by western blotting using anti-NLRX1 antibody.* ***(H)*** *and* ***(I)*** *kinetics of tumor growth in nude mice after injection of RKO cells with hyper expression and stable knockdown of NLRX1 with or without treatment of TNF-α (15 μg/ml) 24 h after administration of cancer cell line.* ***(J)*** *Tumor volume from untreated and TNF-α treated mice was measured every two days. Average tumor volume at day 22 was measured and plotted against each RKO sublines. Data depicts mean ± SEM (n=3). Asterisk (*) indicates that p value < 0.05, for SEM.*

To extend these cellular finding to *in vivo* models, we used RKO colon carcinoma cells to perform xenograft tumor formation assays in nude mice. RKO cells expressing NLRX1 shRNA1 or ectopic full length NLRX1 constructs were injected into nude mice and the growths of subcutaneous tumors were examined every two days (Fig. 5.5H). In agreement with our *in vitro* studies, NLRX1 hyper expression negatively affected the tumorigenic potential of RKO in nude mice. In contrast, NLRX1 knockdown substantially increased the tumorigenic potential of RKO cells. Downregulation of NLRX1 significantly increased tumor volume, as well as the increased efficiency of tumor initiation. Similarly, hyper expression of NLRX1 resulted in smaller tumor volume. The effects were significantly altered if mice received intraperitoneal injections of TNF-α, after a subcutaneous inoculation of RKO cells. TNF-α treatment decreased the tumorigenic potential in all three RKO sublines producing the smallest average tumor volume in NLRX1 expressing cancer cells (Fig. 5.5I and J). *In vivo* data from nude mice with NLRX1 hyper expression and NLRX1 knockdown in RKO cells correlated with *in vitro* data from MCF-7 cell line both in untreated and TNF-α treated condition. However, data with control mice in TNF-α treated condition does not comply with the in vitro study. This could be due to difference in cell lines used for the *invivo* study as both the cells differ in origin and have different tumorigenic capability. These observations suggest that NLRX1 may act as a potential tumor suppressor which negatively regulates the tumorigenic potential of cancer cells**.**

5.6 Discussion:

In the present study, we demonstrated that NLRX1 sensitizes TNF-α-induced cell death by promoting the activation of caspase-8.The crosstalk between NLRX1 and caspase-8 is important for regulation of mitochondrial function and ROS generation during TNF-α-induced cell death. We conclude that the NLRX1 acts as a potential tumor suppressor by modulating TNF-α-regulated mitochondrial function and apoptosis in cancer cells.

Here, we demonstrated that NLRX1 specifically sensitizes TNF-α induced cell death by promoting caspase-8 activation through its association with pro-apoptotic complex-II in various cancer cells of different origin. The exact composition and regulation of the assembly of complex-II during the TNF-α induced apoptosis is not well understood. The analysis of data obtained by monitoring cell death and immunoprecipitation experiments demonstrated the association of NLRX1 with caspase-8 and TRAF2 during TNF-α-induced formation of complex-II. The recruitment of different Ub E3 Ligases (Gonzalvez et al., 2012; Jin et al., 2009) at this step may ubiquitinate NLRX1 (Xia et al., 2011) and regulate the assembly of complex and caspase-8 activation, hence sensitizing the cells to the TNF-α induced cell death.

Caspase-8 acts as a multifunctional protease and translocates to various sub-cellular locations during stress conditions. For example, during ischemia caspase-8 localizes to nucleus and cleaves PARP2 (Benchoua et al., 2002). Previous reports demonstrated the translocation of TNF-α-induced complex-II to mitochondria including casapse-8, TRAF2 and RIP1 during apoptosis (Kim et al., 2010). The evidences presented in the current study suggest that NLRX1 may regulate the association of active caspase-8 with mitochondria in the presence of TNF-α. NLRX1 may regulate the formation of pro-apoptotic complex-II and promote caspase-8 activation at the mitochondria to control OxPhos function in the presence of TNF-α. This observation is in agreement with earlier report demonstrating that caspase-8 may form a native macromolecular complex with Bid at the mitochondria (Gonzalvez et al., 2008), although the functional significance of the association was not understood. Our results suggested that the crosstalk between NLRX1 and caspase-8 is important for the regulation of mitochondrial ROS generation, as the inhibition of caspase-8 activation and NLRX1 depletion both attenuated TNF-α-induced mitochondrial

ROS. Dysregulation of redox balance in response to TNF-α results in leakage of electrons from complex I and complex III to molecular oxygen, thus serving as primary source of superoxide anion radical. The association of mitochondria-localized NLRX1 with activated caspase-8 suggested that proteolytic activity of caspase-8 may regulate the enzymatic activity of mitochondrial respiratory complex I and complex III. A previous report also suggested that NDUFS1, a 75 kDa protein of complex I subunit is a substrate of caspase-3 and is a critical event of mitochondrial dysfunction during apoptosis (Huai et al., 2013). These evidences indicated a caspase mediated regulation of OxPhos system during stress conditions. Similarly, a recent report suggested that casapase-8 is an important bioenergetic determinant and a key regulator of cellular ATP levels (Lanning et al., 2014).The results from our study suggested that NLRX1 may regulate the caspase-8 activation on mitochondria in the presence of TNF-α. The activated pool of caspase-8 associated with mitochondria may cleave the subunits of mitochondrial respiratory complex I or complex III. The association of NLRX1 with UQCRC2, an integral member of the complex III also known as the bc1 complex, is consistent with the hypothesis (Arnoult et al., 2009). The decrease in activity of complex III along with increased levels of mitochondrial ROS observed in NLRX1-tranfected cells in the presence of TNF-α suggested that activated caspase-8 may target components of complex III. However additional studies are required to investigate the association of NLRX1 and caspase-8 with mitochondrial complexes I and complex III and their probable substrates.

Cancer cells within TME, generally exhibit a high level of intrinsic ROS generation and constitutive NF-κB activation. The increased ROS generation coincides with upregulated glucose metabolism under hypoxic conditions, a phenomenon characteristic of fast proliferating cancer cells (He and Karin, 2011; Hsu and Sabatini, 2008). The increased activity of mitochondrial complex I and complex III and elevated ATP levels observed inNLRX1-KD cells in the presence of TNF-α suggested an increase in mitochondrial OxPhos capacity and energy production. This assumption is supported by a previous report showing that TNF-α regulate the expression of key metabolic enzymes, such as glycogen phosphorylase (PYGL) and glutamate dehydrogenase 1 (GLUD1). PYGL increases the flux of glucose from glycogen reserves whereas GLUD1 activity provides the substrates for anaplerotic metabolism (Zhang et al., 2009). We demonstrated that ectopic ex-

pression of NLRX1 in cancer cells inhibits the activity of mitochondrial respiratory chain complexes, generates ROS and sensitizes them to TNF-α induced cell death. Altogether, these results indicated that NLRX1 has a tumor suppressor activity. In ER and PgR positive breast carcinoma cells, MCF-7 and T47D, NLRX1 expression is downregulated. We show that the ectopic expression of NLRX1 in MCF-7 cells compromised clonogenic ability of these cell. Similarly, ectopic expression of NLRX1 in RK cells, a colon carcinoma cell line, decreased *in vivo* tumor formation in a nude mice xenograft assay. The increased levels of TNF-α observed in tumor microenvironment and loss of NLRX1 expression may facilitate the metabolic reprogramming to meet the anaplerotic demands in these cancer cells. The higher expression of NLRX1 was observed in ER and PgR negative MDA-MB-231, a highly metastatic cell line and HBL-100, a SV40 mediated immortalized cell line. This strongly suggests that NLRX1 may have a different role in an altered tumor microenvironment. Recent evidence suggests that aggressive and metastatic property of MDA-MB-231 cells is attributed to autophagy-dependence. These cells are addicted to autophagy for the survival even in nutrient rich conditions (Maycotte et al., 2014). Similarly, previous reports suggest that NLRX1 acts as a positive regulator of autophagy during the antiviral signaling (Lei et al., 2012). Altogether, these evidences raise the possibility that the up-regulated expression of NLRX1 may synergistically regulate metabolism and autophagy for highly invasive growth of the autophagy addicted MDA-MB-231 breast cancer cells. Therefore, the tumor suppressor role of NLRX1 may be cell type dependent and will be highly specific to the tumor microenvironment. The heterogeneity observed in the breast cancer cells in tumor microenvironment further strengthens the hypothesis (Polyak, 2011). The responsiveness of these cell lines to TNF-α induced cell death as well metabolic reprogramming needs additional studies.