Autophagy in the Etiology of Acute Myeloid Leukaemia with Nucleophosmin Mutation

Tan Hwei Ee[1], Tan Ban Xiong[2], Leong Sai Mun[2], Lim Tit Meng[2]

[1]SRP student, Hwa Chong Institution (College)
[2]Department of Biological Sciences, National University of Singapore

Abstract

Acute myeloid leukaemia (AML) is a cancer associated with the accumulation of immature white blood cells which thus interferes with the development of normal blood cells, sprouting a whole array of symptoms such as anaemia. Like other types of cancer, resistance to apoptosis, or programmed cell death, is seen with AML (Hanahan et al., 2000). In one-third of primary AML patient specimens, cytoplasmic localisation caused by a mutation of the normally nuclear nucleophosmin (NPM) is observed (Falini et al., 2005). NPM normally functions to prevent nucleolar protein aggregation, regulate assembly and nucleocytoplasmic transport of pre-ribosomal particles, and regulate the ARF-p53 tumour-suppressor pathway. However, the exact cytoplasmic role of cNPM is unknown. Previous results in our laboratory have demonstrated that cNPM specifically inhibits caspases-6 and -8, which are part of apoptotic activation signalling. Interestingly, expression of ATG12, which is involved in autophagosome formation, was elevated as well with cNPM overexpression, thereby implying increased autophagy, or cellular self-digestion, which is a cellular pathway involved in protein and organelle degradation. In pursuant of current line of evidences, this study investigates the extent of autophagosome formation with mutant cNPM overexpression. More autophagosomes are observed in cells with cNPM as compared to cells with normal wild type NPM. The results also show that inhibition of caspases-6 and -8 promotes autophagy. In conclusion, the results of this study suggest that increased autophagy brought about by inhibition of caspases by cNPM ultimately contributes to cell death resistance in AML.

Introduction

In normal individuals, production of white blood cells in the bone marrow is orderly and regulated. However, in acute myeloid leukaemia (AML), this process gets out of control and many abnormal and immature white blood cells (myeloblasts) that do not function normally are produced. This is due to genetic mutations that arrest proper cellular differentiation. Combined with other mutations, proper regulation of cell growth and proliferation is also lost. The malignant proliferation causes accumulation of myeloblasts in the bone marrow, taking up space that is needed to make normal red and white blood cells. Hence, anaemia results, as well as an increased risk of infection. If not treated, acute leukaemia can be perpetually fatal, usually because of complications involving infiltration of myeloblasts into vital organs. Even with treatment, chances of full recovery vary. 300 new cases of leukaemia are diagnosed each day in the United States (The Leukemia and Lymphoma Society Web Site, 2004). and the average survival time for AML patients is only 1 year after diagnosis, even with aggressive treatment.

Resistance to apoptosis is often seen with cancer, such as in AML (Hanahan et al., 2000). Apoptosis, or programmed cell death, is the process where cells die in response to stimuli in a controlled manner. Apoptosis may function to remove damaged, abnormal, or unnecessary cells, thereby maintaining the balance between cell proliferation and cell death.

There are several pathways by which induction of apoptosis may occur, two of which are well characterised: the extrinsic apoptotic pathway, also known as the death receptor pathway, or the intrinsic pathway, also known as the mitochondrial pathway (Kroemer *et al.*, 2007, Riedl & Salvesen, 2007, Youle and Strasser, 2008). The main executors of the apoptotic process are enzymes called caspases. In any case, both pathways ultimately converge at the apoptotic caspase cascade, and the cell typically dies in a manner of caspase-dependent cell death (CDCD). Apoptotic signalling by interactions among initiator caspases such as caspase-8 and effector caspases such as caspase-6 is implicated in CDCD.

CDCD may be inhibited by inactivation of caspases, but even so, evasion of cell demise is not guaranteed as caspase-independent cell death (CICD) may occur. CICD utilises alternative means, and may be brought about by production of reactive oxygen species, loss of mitochondrial function or released mitochondrial intermembrane space proteins such as apoptosis-inducing factor or endonuclease G to catalyse death (Chipuk *et al.*, 2006).

Nucleophosmin (NPM), a protein that shuttles between the nucleus and cytoplasm, should normally be localised in the nucleus, and is especially predominant in the nucleolus. NPM normally functions as a molecular chaperone that may prevent protein aggregation in the nucleolus and regulate the assembly and transport of pre-ribosomal particles through the nuclear membrane. NPM also regulates the ARF-p53 tumor-suppressor pathway. However, aberrant cytoplasmic localisation of NPM is observed in about 30% of primary AML patient specimens. Primary AML specimens with cytoplasmic NPM (cNPM) have translocations involving the NPM gene that are predicted to alter the protein at its C-terminal and cause its aberrant cytoplasmic localisation (Falini *et al.*, 2005). However, little is known about the precise cytoplasmic role of the mutant cNPM. It was shown that NPM specifically inhibits caspases-6 and -8 (Leong, 2005). This implied that cNPM could also inhibit CDCD through inactivation of caspases. Recent results in our laboratory reported increased amounts of ATG12, a protein required for the formation of autophagosomes for cellular autophagy, and also upregulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH-mediated protection of cells from CICD involved an elevation in glycolysis and increased in ATG12 expression, which enhanced autophagy, and as a result tends to preserve survival by removing defective mitochondria (Colell *et al.*, 2007). Moreover, autophagy is found to preserve cell survival in metazoans by conferring metabolic benefits to cells under stress (Lum, 2005). Thus, it seems probable that cNPM could have also inhibited CICD by enhancing autophagic processes, and this has implications for cell survival and oncogenesis.

Present evidences seem to suggest that the role of mutant cNPM in the preservation of cell survival in leukaemia is characterised by the inhibition of both CDCD, by inactivation of caspases-6 and -8, and CICD, by promoting autophagy. Hence, the aim of this study is to investigate, by western blotting method and fluorescence confocal imaging, firstly: the extent of autophagosome formation with mutant cNPM overexpression, and secondly: the relationship between autophagy and inhibition of caspases-6 and -8. We hypothesised that more autophagosomes will be observed in cells with cNPM as compared to cells with normal wild type NPM, and inhibition of caspases-6 and -8 promotes autophagy.

Materials and methods

Cell culture: Human embryonic kidney (HEK) 293T cell lines (gift from A/P Low Boon Chuan, NUS) were cultured in RPMI 1640 (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) in a 10 cm culture plates. Cell lines were maintained within a humidified atmosphere of 5% CO_2 at 37°C and passaged at 90% confluence.

Transfection and drug treatment: Cells were seeded on for 2 cm 6-well plates for transfection and drug treatment. Samples for microscopy were grown on cover slips placed in the wells. Transfection of previously prepared pcDNA3.1 plasmids containing gene constructs for *Aequorea victoria* GFP (AvGFP), AvGFP tagged to wild type NPM (GFP-wtNPM), AvGFP tagged to cytoplasmic mutant NPM (GFP-cNPM) (Leong, 2005), into HEK 293T cell line was performed using Lipofectamine 2000 (Invitrogen, USA) following manufacturer's instructions, using 5 µg DNA and 2.5 µl Lipofectamine 2000 reagent. In an optimised repeat, *Aequorea coerulescens* GFP1 (AcGFP1) was used instead of *Aequorea victoria* GFP for all 3 gene contructs. Samples for microscopy were additionally co-transfected with 0.5 µg red fluorescent protein microtubule-associated protein 1 light chain 3 (RFP-LC3) and 2.5 µl Lipofectamine 2000 reagent. Drug treatment was performed 6 hours after transfection with 15 µM of caspase-6 inhibitor, caspase-8 inhibitor, or dimethyl sulfoxide (DMSO) as negative control for caspase inhibitor treatment.

Laser confocal microscopy and puncta analysis: 30 hours after transfection, culture wells were rinsed with PBS, and HEK293T cells were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. The cells were then washed with PBS and stained with Hoechst 33324 stain for 5 minutes. The stain was washed off with PBS and the cover slips with the cells were mounted onto glass slides with Dakocytomation fluorescence mounting media (Dako, USA). Laser confocal micrographs were obtained with LSM 510 Meta confocal microscope (Zeiss, Germany). The images were analysed using ImageJ software (NCBI, USA), where thresholding was performed with the segmentation plugin (McMaster Biophotonics Facility, Canada) and RFP-LC3 puncta larger than 0.0225µm^2 were quantified.

Electrophoresis and western blotting analysis: Culture wells were rinsed with PBS 30 hours after transfection. Then cells were lysed with total cell lysis buffer (100 mM HEPES pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% volume by volume (v/v) protease inhibitor cocktail). Cells were dislodged and harvested with a cell scraper, and incubated on ice for 10 minutes with regular vortexing. The lysates were quantified using Brandford assay, and equal amounts are loaded with SDS PAGE buffer and heated to 95^{o}C for 5

min. Proteins were separated under denaturing conditions in 15% polyacrylamide gels and electroblotted onto nitrocellulose membranes at 90V for 90 min. Membranes were blocked with 3% weight by volume (w/v) skimmed milk for 15 min in Tris buffered saline with 0.1% v/v Tween-20 (TBST) followed by overnight incubation with primary antibodies. Primary antibodies used were anti-β-actin (Sigma, USA, 1:10000) and anti-LC3 (Cell Signaling Technologies, USA, 1:1000). The blots were then washed with 3 washes with TBST of 5 min each, and incubated with HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, USA). Protein bands were visualised with Pierce Biotechnology SuperSignal West Pico and Femto chemiluminescent substrates following manufacturer's recommendations and exposed onto CL-X Posure films (Pierce Biotechnology, USA). Densitometry was performed by first digitising the films with a flatbed scanner, and then the area and mean gray value of the bands were analysed with ImageJ software.

Results

Localisations of cNPM and wtNPM in the cell are verified by laser confocal microscopy. GFP fluoresce green when viewed under laser confocal microscopy, so observing the GFP fluorescence by laser confocal microscopy allows visualisation of the localisation the respective proteins: GFP for cells that have been transfected with GFP; mutant NPM for cells that have been transfected with GFP-tagged mutant NPM (cNPM); wild type NPM for cells that have been transfected with GFP-tagged wild type NPM (wtNPM). Nuclei is visualised with Hoechst stain which specifically stains DNA. Within the nucleus, small circular areas with absence of blue fluorescence demarcate the nucleoli. The nucleolus is not stained as DNA is absent in the nucleolus. In both Figures 3.1.1-A and 3.1.2-A, GFP gene is not tagged to cNPM or wtNPM. Green fluorescence is distributed throughout the entire cell, with no localisation in any specific areas of the cell. This affirms the expected behaviour of GFP. By comparing the green GFP fluorescence against the blue Hoeschst fluorescence between rows B and C in both Figures 3.1.1 and 3.1.2, it is evident that mutant cNPM was localised in the cytoplasm, whereas wtNPM had normal localisation in the nucleus, or more precisely, the nucleolus. This affirms the expected activities of mutant cNPM and normal wtNPM following successful transfection of HEK 293T cells. It is notable that the localisation characteristic of cNPM and wtNPM is especially distinct in cells transfected with *Aequorea coerulescens* GFP1 (AcGFP1) gene constructs, as represented in Figure 3.1.2. In cells transfected with GFP-cNPM, cytoplasmic localisation of NPM as indicated by green fluorescence is not evident in as many cells as expected. The difference between both repeats suggests that pcDNA3.1 plasmids containing gene constructs for *Aequorea victoria* GFP (AvGFP) tagged to NPM may have interfered with the localisation of NPM as GFP-NPM oligomers are formed. AcGFP1 was derived from the jellyfish *Aequorea coerulescens* and is a true monomeric protein, hence AcGFP1 poses as a more ideal candidate for fusion tag applications such as NPM tagging. The optimised repeat with AcGFP1 plasmids may therefore confer additional certainty in the results, atop the experimental results using AvGFP plasmids. The above observations agree with the study by Falini et al. in 2005, espousing that the altered C-terminus of NPM caused by the translocation mutation resulted in aberrant nuclear export results in cytoplasmic localisation.

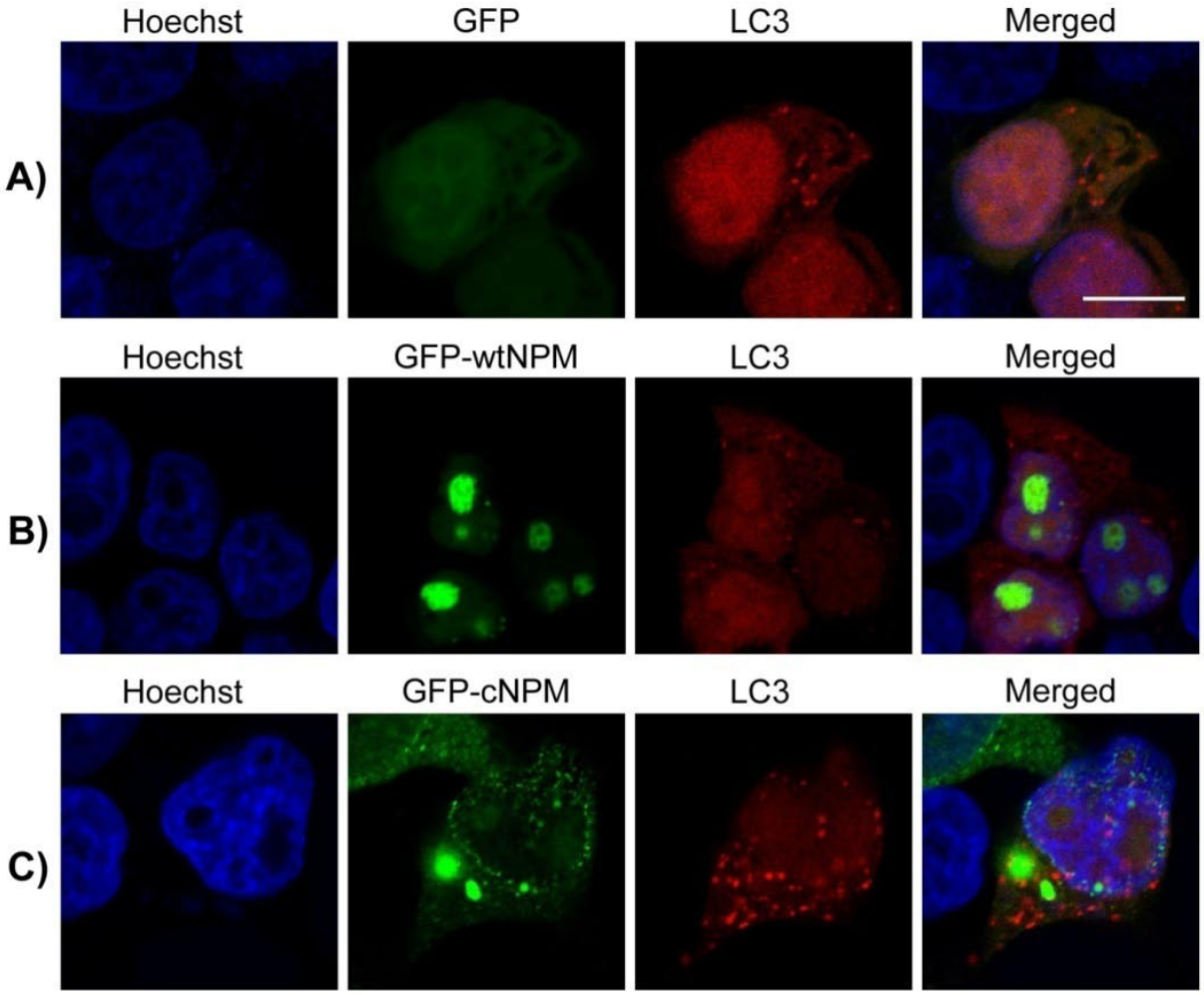

Figure 3.1.1 Representative fluorescent micrographs of HEK 293T cells co-transfected with RFP-LC3 and either **A)** GFP, or **B)** GFP-wtNPM, or **C)** GFP-cNPM, all of *Aequorea victoria* GFP gene constructs. The scale bar represents 10μm.

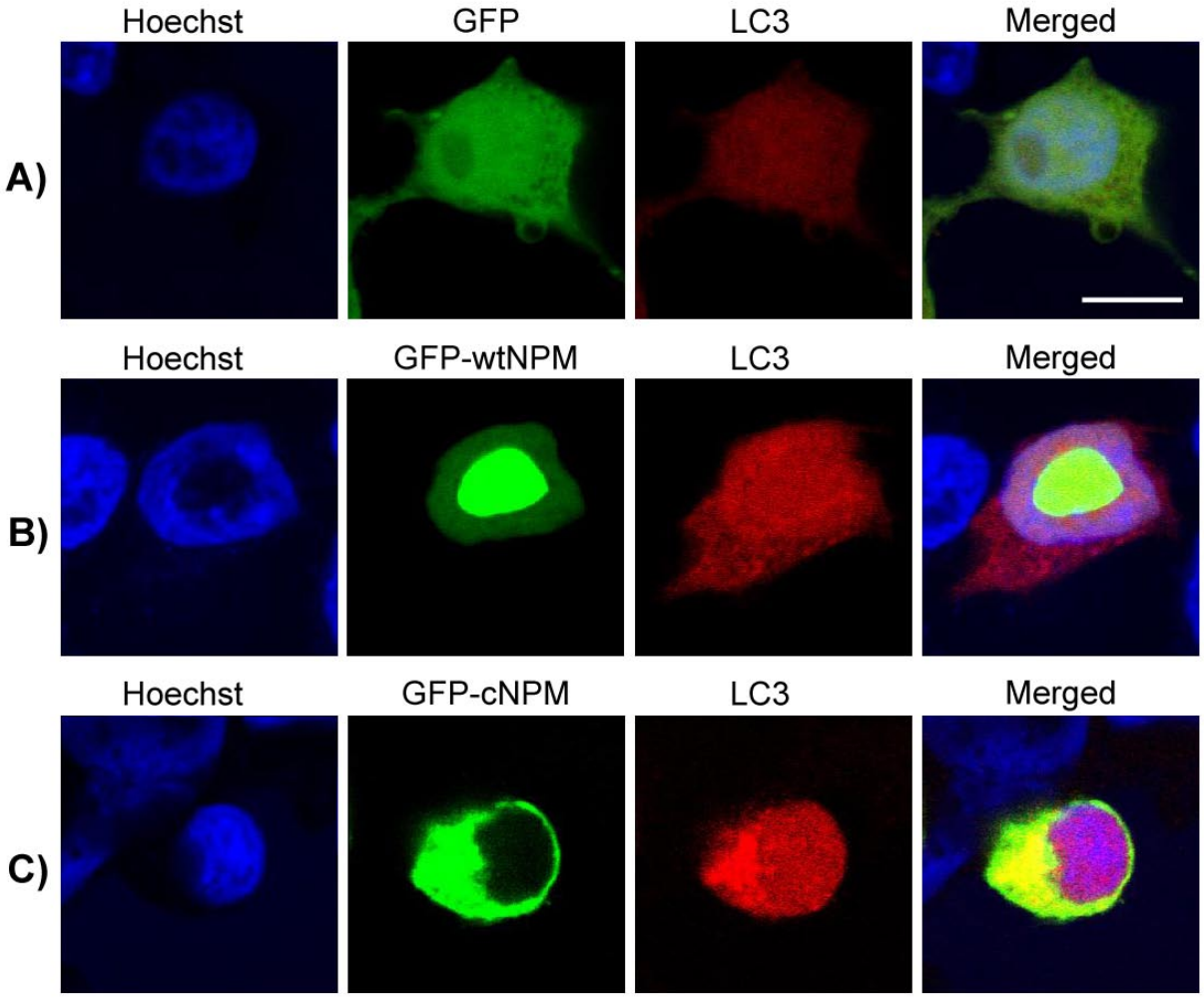

Figure 3.1.2 Representative fluorescent micrographs of HEK 293T cells co-transfected with RFP-LC3 and either **A)** GFP, or **B)** GFP-wtNPM, or **C)** GFP-cNPM, all of *Aequorea coerulescens* GFP1 gene constructs. The scale bar represents 10μm.

Overexpressing mutant cNPM promotes autophagy. To expound the link between abnormal cytoplasmic localisation of cNPM and resultant levels of autophagy, RFP-tagged microtubule-associated protein 1 light chain 3 (RFP-LC3) is co-transfected with, separately: GFP, GFP-wtNPM, and GFP-cNPM. Under laser confocal microscopy, RFP-LC3 gene products fluoresce red. LC3 is involved in formation of autophagosomes during autophagy (Tasdemir *et al.*, 2008), and thus poses as a viable autophagic marker. LC3 congregates at sites of autophagosome formation. These clusters of LC3 that are indicative of autophagosomes, being tagged with RFP, can be visualised as red dots by laser confocal microscopy. Transfection of HEK 293T cells with RFP-LC3 allows autophagosome formation activity to be observed. Higher levels of autophagy can be inferred if more autophagosomes are observed. ImageJ software was used for the analysis and quantification of puncta. For each variable, the average number of puncta per cell and average area of puncta per cell were evaluated from randomly picked cells. Only puncta of size greater than 0.025 μm^2 were considered as smaller puncta are unlikely to be autophagosomes (Klionsky, 2000) and may simply be background signals picked up by the LSM 510 Meta detector. With reference to representative fluorescence micrographs, Figures 3.2.1 (of cells transfected with *Aequorea victoria* GFP gene constructs) and 3.2.2 (of cells transfected with *Aequorea coerulescens* GFP1 gene constructs) show that GFP-cNPM transfected cells clearly have about 2 times more puncta as compared to GFP and GFP-wtNPM transfected cells on average. Marked increased puncta area is observed with mutant cNPM as shown in Figures 3.2.3 (of cells transfected with *Aequorea victoria* GFP gene contructs), but not so much in Figure 3.2.4 (of *Aequorea coerulescens* GFP1 gene constructs). Lastly, it is also worth noting that as presented in Figure 3.2.1, 4 cells out of 26 random replicates (15.4%) of cNPM have number of puncta within the upper percentile of 16 to 36, whereas only 1 cell out of 26 random replicates (3.8%) have number of puncta in the upper range. Similarly, Figure 3.2.2 shows similar trend, with 3.0%, 9.1% and 24.2% for GFP, wtNPM and cNPM transfected cells. Cells with the most puncta were singled out for each condition and are presented in

Figure 3.2.5. These results showing a much higher frequency of cells with a lot of puncta observed with cNPM as compared to GFP and wtNPM reveals compelling evidence that cNPM promotes autophagy.

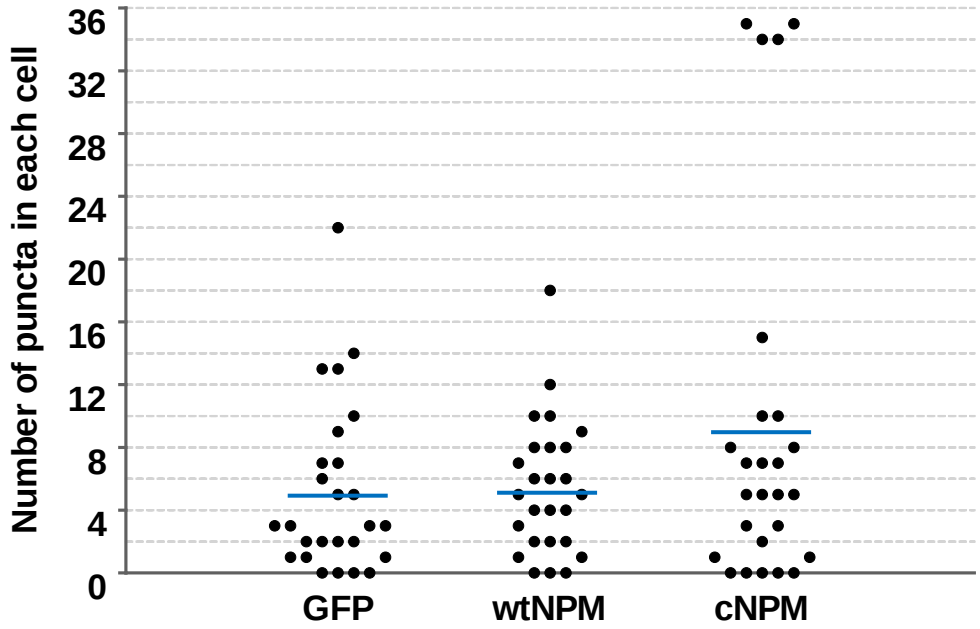

Figure 3.2.1 Scatter graph of the all the replicates of each variable (cells transfected with GFP, GFP- wtNPM or –cNPM, of *Aequorea victoria* GFP gene constructs). A total of 26 random cells were sampled for each treatment. The horizontal lines denote the mean number of puncta per cell among the 26 replicates for each variable. GFP transfected cells have an average of 5.15 puncta per cell, while GFP-wtNPM has 5.42, and GFP-cNPM has 9.23.

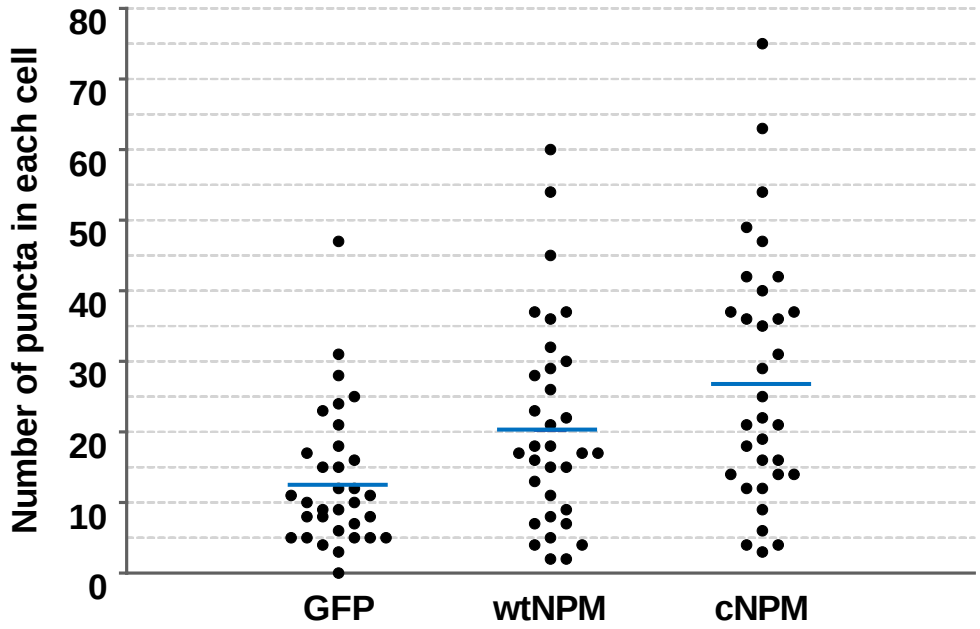

Figure 3.2.2 Scatter graph of the all the replicates of each variable (cells transfected with GFP, GFP- wtNPM or –cNPM, of *Aequorea coerulescens* GFP1 gene constructs). A total of 33 random cells were sampled for each treatment. The horizontal lines denote the mean number of puncta among the 33 replicates for each variable. GFP transfected cells have an average of 13.12 puncta per cell, while GFP-wtNPM has 20.76, and GFP-cNPM has 27.36.

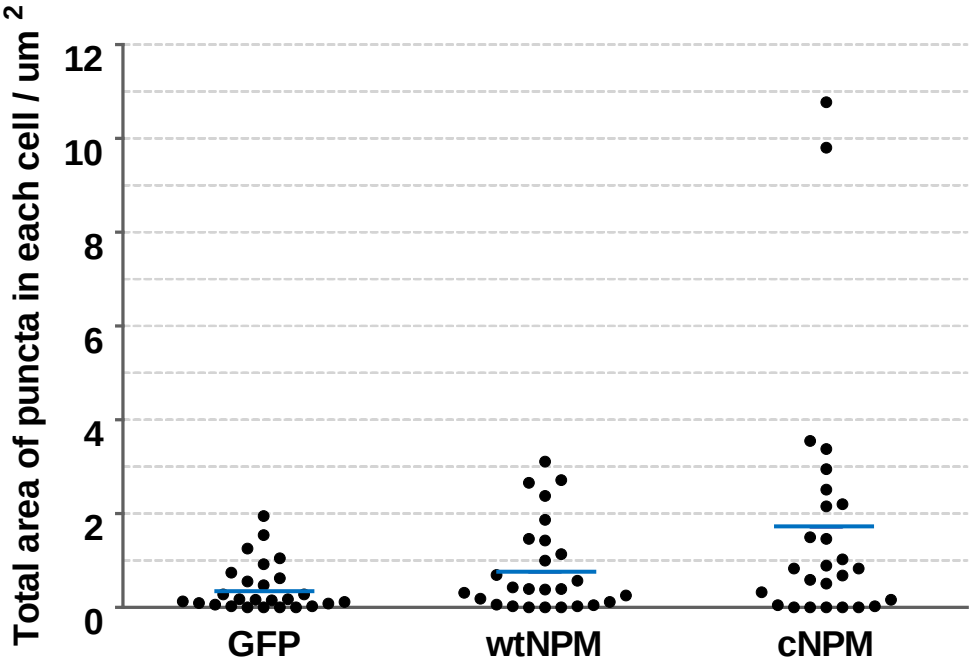

Figure 3.2.3 Scatter graph of the all the replicates of each variable (cells transfected with GFP, GFP- wtNPM or –cNPM, of *Aequorea victoria* GFP gene constructs). A total of 26 random cells were sampled for each treatment. The horizontal lines denote the mean total area of puncta per cell among the 26 replicates for each variable. The average of the total area of puncta per cell for GFP transfected cells is 0.42 μm^2, while GFP-wtNPM is 0.83 μm^2, and GFP-cNPM is 1.78 μm^2.

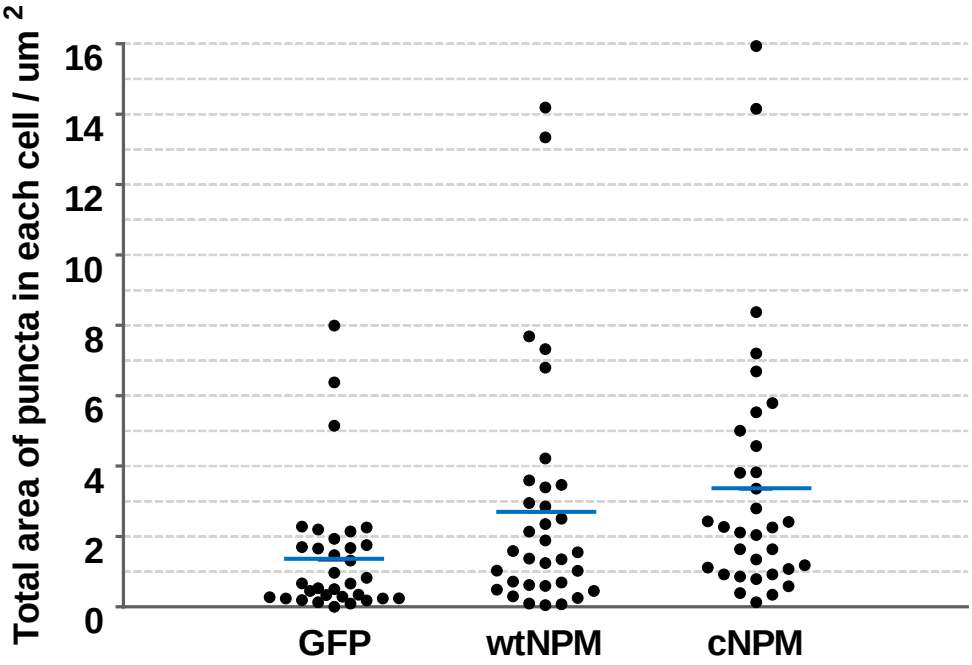

Figure 3.2.4 Scatter graph of the all the replicates of each variable (cells transfected with GFP, GFP- wtNPM or –cNPM, of *Aequorea coerulescens* GFP1 gene constructs). A total of 33 random cells were sampled for each treatment. The horizontal lines denote the mean total area of puncta per cell among the 33 replicates for each variable. The average of the total area of puncta per cell for GFP transfected cells is 1.43 μm^2, while GFP-wtNPM is 2.79 μm^2, and GFP-cNPM is 3.44 μm^2.

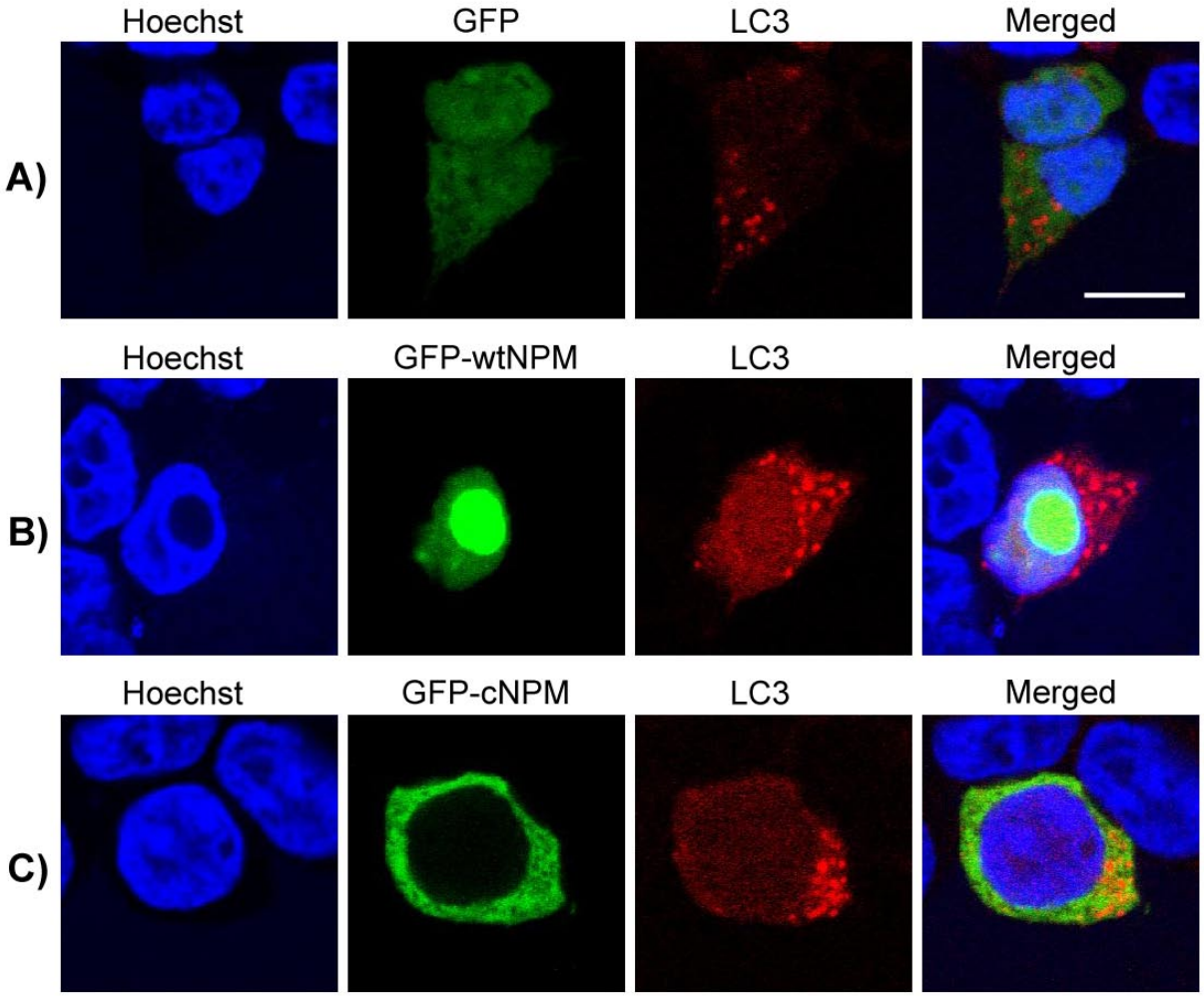

Figure 3.2.5 Fluorescent micrographs featuring cells with extremely high number of LC3 puncta. Images are of HEK 293T cells co-transfected with RFP-LC3 and either **A)** GFP, or **B)** GFP-wtNPM, or **C)** GFP-cNPM. The scale bar represents 10μm.

cNPM promotes autophagy by inhibition of caspases-6 and -8. Autophagic activity is compared among GFP transfected HEK 293T cells under no treatment, under treatment with chemical inhibitor of caspase-6, and under treatment with chemical inhibitor of caspase-8, and cNPM transfected cells. Separation by SDS PAGE and western blotting allows analysis of both the unconjugated (LC3-I) and conjugated (LC3-II) forms of endogenous LC3. As one of the processes in autophagy, LC3-I in the cytoplasm is converted into phosphatidylethanol-conjugated LC3-II form localised on the isolation membrane and the autophagosome membrane. Hence, by comparing the ratio of LC3-II to LC3-I, autophagosome formation can be monitored, and thus the level of autophagy can be deduced. By comparing the GFP and cNPM columns in Figure 3.3.1 and quantitative densitometric analysis in Figure 3.3.2, it is clear that that cells that were transfected with cNPM had upregulation of LC3-II in relation to LC3-I, as compared to cells that were transfected with GFP. The results show that GFP transfected cells treated with caspase-6 inhibitors have greatly increased levels of autophagy that are closely comparable to that of cNPM transfected cells. Inhibitor of caspase-8 however, appears to have little effect on autophagy as the ratio of LC3-II to LC3-I is generally similar to that in GFP transfected cells.

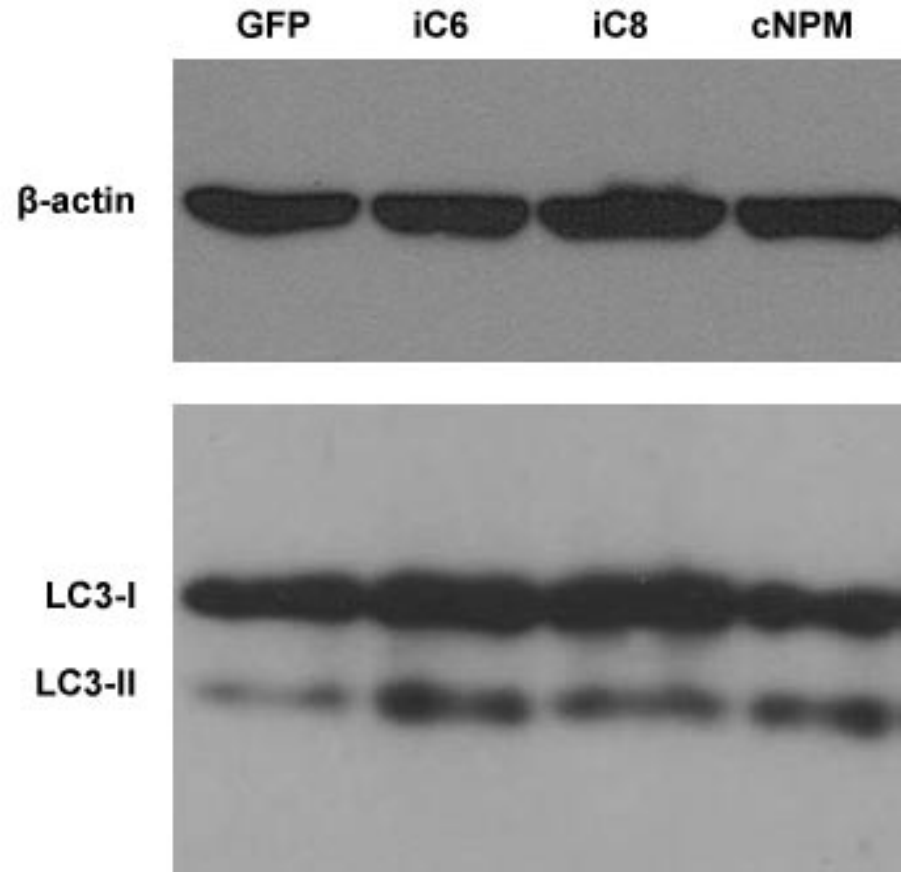

Figure 3.3.1 Western blot showing relative β-actin, LC3-I, LC3-II levels. Samples in the GFP, iC6 and iC8 columns are all from cells transfected with GFP, differing in that samples in the GFP column are of cells treated with DMSO, and samples in iC6 and iC8 columns are from cells treated with inhibitors of caspases-6 or -8 respectively. Blots in the cNPM columns are from cells transfected with cNPM, and treated with DMSO similarly as a negative control for addition of caspase inhibitors. *Aequorea coerulescens* GFP1 gene constructs gene constructs were used for all 4 samples. Analysis of loading control, β-actin, showed equal protein loading.

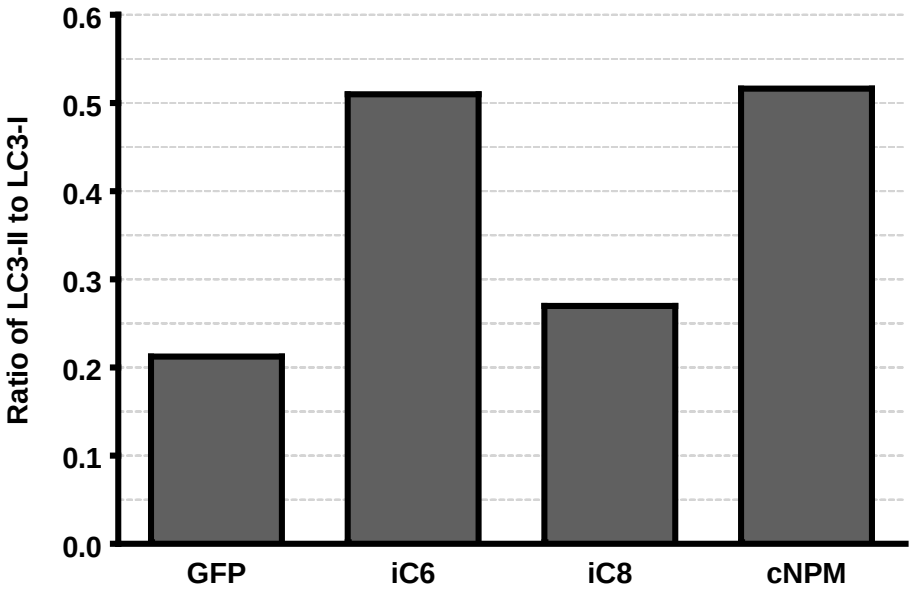

Figure 3.3.2 Bar graph displaying ratio of the relative intensities of LC3-II to LC3-I for each of the 4 samples. Relative intensities of the 8 bands among one another were derived by densitometric analysis of the LC3 blot shown in Figure 3.3.1.

Discussion

The number of autophagosomes may serve as a more reliable indicator for assessing the extent of autophagy than the size of autophagosomes. Going by RFP-LC3 puncta count, hence the number of autophagosomes in the cell, as an indicator of autophagy levels, cNPM clearly ramps up levels of autophagy in both repeats, however, going by puncta area, hence total size of autophagosomes in a cell, the trend, while still true, is not sufficiently distinct to be convincing. From the results, it appears that total area of puncta per cell may not be reflective of the extent of autophagy after all. As such, the subtle trend observed when autophagosome size is used to characterise extent of autophagy may simply be due to the trend in the number of puncta, as cells with more number of puncta naturally tend to have a greater total area of puncta. The relationship between puncta area and autophagic activity is also similarly contested by Klionsky et al. in a comprehensive review of assays for monitoring autophagy.

The pro-survival function of cNPM appears to first take effect at the apoptotic caspase cascade. Inhibition of caspases-6 and -8 by cNPM effectively circumvents programmed cell death of leukaemic cells irregardless of whether the apoptotic trigger is extrinsic (activated by ligation of external ligands to death receptors) or intrinsic (release of cytochrome *c* from the mitochondria) as both pathways converge at the caspase cascade.

However, with caspase-dependent cell death (CDCD) inhibited, caspase-independent cell death (CICD) may still proceed. Indeed, it is well documented that inhibition of the apoptotic caspase cascade, tends the cell towards alternative death pathways resulting in excessive autophagy, or necrosis (Vandenabeele, 2006 and Bell, 2008). According to Vandenabeele, necrotic and autophagic cell death seems to be closely related and intermingling processes. In summary, 3 main outcomes are plausible following CDCD subversion: First, autophagic cell death may first start as a survival attempt, blocking necrosis and removing oxidatively damaged mitochondria. When this process occurs in excess, autophagy itself becomes cytotoxic and eventually leads to autophagic cell death. Otherwise, autophagy as a subversion attempt fails, and death by necrosis still occurs. The third plausible outcome is that attempts at cell survival by protective autophagy turns out successful – cell death is thereby entirely circumvented.

In the case presented herein, increased autophagy is an effect of mutant cNPM, which is characteristically prevalent in one-third of AML cases. This implies that the increased autophagy linked to cytoplasmic localisation of the mutant protein should be a leukaemogenic feature, and contributes to the ability of leukemic cells to evade and resist cell death. Furthermore, the protective function of autophagy has also been elucidated by Han et al. in 2008, who verified the involvement of protective autophagy in tumour cells. Moreover, the notion that autophagy leads to cell death instead of resisting cell death is highly contested in recent literature, drawing the distinction between occurrence of autophagy that simply coincides *with* cell death, and autophagic cell death which is autophagy as a *cause* of cell death (Kromer & Levine, 2008), espousing the pro-survival role of autophagy as proposed by Colell et al., Lum et al., Tan, and Leong. In summary, while 2 conflicting views of autophagy in relation to cell death have been proposed in current literature, in the specific context of this study that concerns autophagy in AML with NPM mutation, to theorise that the consequent increase in levels of autophagy is tied to autophagic cell death instead of survival conflicts with the dominant discourse, and goes against evidences presented in the current sphere of literature.

Results reveal that cells with overexpression of cNPM have a much higher average number of autophagosomes per cell than with wtNPM overexpression. This interpretation may be compounded with an alternative interpretation going by the assessment of peak values. A higher frequency of occurrence of cells with a lot of autophagosomes in the experimental set of cells overexpressed with cNPM, than in cells overexpressed with wtNPM is observed. It is highly probable that these cells are ultimately the ones responsible for malignancy and leukaemogenesis. More cells displaying higher than usual levels of autophagy in the cNPM set implies that it is more likely that one of them may eventually successfully develop resistance to cell death. Unregulated proliferation characterised by resistance of cell death in just a single cell, if persisted, may result in development of tumours, and if further aggravated by angiogenesis and metastasis, may eventually develop into malignant cancer. With marked increase in level of autophagy in cNPM cells, it is much more likely for a cNPM cell to develop resistance and malignancy as compared to wtNPM cells.

Figure 4 displays the biological pathway linking key points of this study. In summary, the results suggest that cNPM promotes leukaemogenesis is two main ways: Firstly, through inhibition of caspases-6 and -8 thereby disrupting the apoptotic caspase casade prior to CDCD, and secondly, through inhibition of caspase-6 thereby promoting autophagy to subvert CICD, such as necrosis, ultimately preserving cell survival.

It may be worth nothing that inhibition of caspase-6 is shown to prevent both CDCD and CICD, whereas caspase-8 inhibition is only implicated in the prevention of CDCD. The findings in this paper therefore suggest that caspase-8 is less directly involved in autophagy regulation unlike caspase-6. Interestingly, caspase-8 has been hailed as a critical regulator that is involved in mediation of the entire death spectrum of apoptosis, autophagy and necrosis (Vandenabeele, 2006). This apparent contradiction leaves a lot of potential for further investigations into the precise role of caspase-8 in cell death and survival.

Based on the results of this paper, there is exciting potential for future studies to examine treatment strategies for AML, to counteract the anti-apoptotic and pro-survival function of mutant cNPM. The most direct strand of future research would be to ascertain the outcome of autophagy as observed in this paper by monitoring autophagic flux from the initial formation of autophagosomes to the digestion of cellular components or organelles. Autophagy assessment is hailed as considerably contentious issue. There is no one single definitive way of assessing autophagy levels in cells. Western blotting, fluorescence imaging, electron microscopy – the three dominant ways used by the majority of studies to assess autophagy – all have their characteristic flaws (Tasdemir et al., 2008). Fundamentally, the common notion that greater number and size of autophagosomes observed indicate higher levels of autophagy is also contested, because there is a difference between measurements that monitor the number of autophagosomes versus those that measure flux through the autophagic pathway. The present results of this study leaves ambiguity as to whether it is a block in macroautophagy that leads to autophagosome accumulation, or whether it is fully functional autophagy that includes delivery to, and degradation within lysosomes. Nonetheless, cNPM being a characteristic feature in one-third of AML patients, implies the increased autophagy linked to cytoplasmic localisation of the mutant protein should be a leukaemogenic feature, and contributes to the ability of leukemic cells to evade cell death. This line of argument is also congruent to the dominant discourse in present scientific literature. This paper suggests that future studies should perform steady-state transfection in lieu of transient transfection of NPM. This would minimise LC3 aggregation thereby improving the accuracy for fluorescence confocal analysis. Secondly, Atg12-Atg5 conjugation monitoring will also give further support to results by SDS PAGE and fluorescence microscopy of LC3 puncta. Thirdly, transmission electron microscopy (TEM), initially planned for this study, will also allow for more direct visualisation of autophagosomes, and will certainly confer even greater certainty when combined with the current pool of evidences. TEM results are however unavailable due to technical issues with the machine. And lastly, with the pathways elucidated in this paper, future studies may feasibly decide to use an AML cell line, avoiding the need for exogenous NPM overexpression.

While this study uses HEK 293T for clarity in investigating the molecular mechanisms of mutant cNPM and autophagy, future studies may focus on emulating actual conditions with AML patient samples or cell lines. Ideally, AML patient samples would serve as highly definitive models for study. However, patient samples are difficult to obtain. As an alternative to using patient samples, human AML cell lines such as K562, KG-1 and HL-60 (Koeffler & Golde, 1980) should similarly serve as definitive models for study. However, these cell lines are not readily transfectable. Moreover, HL-60 or other AML cell lines, being blood cells, have characteristically smaller sized cells than HEK 293T. Due to much smaller cytoplasmic size – i.e. RFP-LC3 puncta could not have been feasibly quantified. In the context of this present study, ease of transfection of cells is a critical consideration as cells would be transfected with various plasmids. In contrast to K562, KG-1 or HL-60, the HEK 293T cell line is highly transfectable. It is also a human cell line, thereby conferring a reasonable degree of genetic similarity with human leukaemic cells. Having clearly mapped out the molecular mechanisms with HEK 293T, further studies may focus on emulating actual conditions with AML patient samples or cell lines.

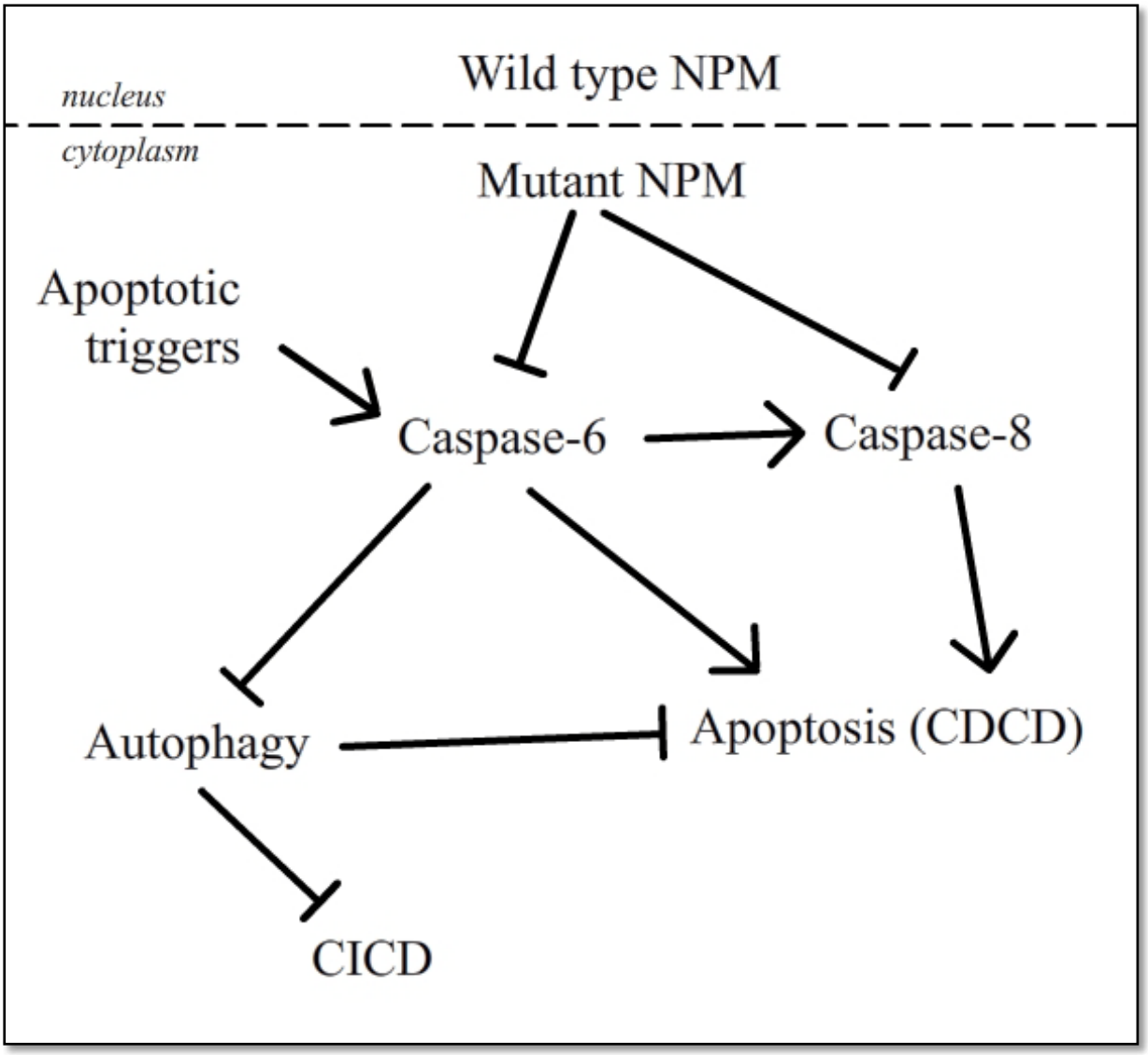

Figure 4 Biomolecular pathway diagram showing the proposed mechanism of mutant NPM in resistance to cell death in AML. The translocation mutation resulting in the nuclear export of NPM may be seen as the start point, triggering a whole myriad of interactions in the cytoplasm that eventually confers resistance to cell death. Without inhibition of caspases-6 and -8, apoptotic signalling is not disrupted, and cell may undergo apoptosis which has the benefit of disallowing mutations to accumulate to the extent that cancer ensues. Inhibition of caspases-6 and -8 thereby naturally prevents apoptosis. On top of prevention of apoptotic CDCD by caspase inhibition, results indicate that inhibition of caspase-6 (but not caspase-8) by cNPM leads to increased autophagosome formation, suggesting heightened levels of autophagy. Metabolic benefits of autophagy and at times, removal of defective organelles by autophagy tend to the conclusion that the increased autophagy observed in this paper is responsible for resisting cell death, by even inhibiting CICD.

Conclusion

The evidences prior to this paper bare one highly significant gap in the understanding of how NPM mutation enables cell death resistance in leukaemogenesis. The results in this paper clearly establish increased autophagy as the missing link, piecing in nicely with prior evidences of caspase-inhibitory effects of cNPM, and pro-survival function of autophagy. With increased autophagic activity identified to be an event downstream of cNPM mutation that contributes to the cell being able avoid cell death, this paper sets the path for future studies which may develop treatments to circumvent leukaemogenesis at the point following cytoplasmic localisation of cNPM.

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