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14. ABSTRACT Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder characterized by the development of brain tumors of peripheral nervous system origin. We endeavored to improve understanding of NF2 pathogenesis at the genetic and molecular levels. The gene causative for NF2 has been identified as the tumor suppressor Merlin, and several mutations of Merlin found in NF2 patients have been linked to loss of growth suppressive activity of Merlin. A major goal of this project has been to elucidate novel functions of Merlin in autophagy, a cellular clearance system responsible for degrading old proteins or damaged organelles within cells, thus helping to mitigate the risk of tumor formation. We successfully confirmed the novel role of Merlin in promoting autophagy. We showed that Merlin is a part of multiprotein complex that serves as a scaffolding machinery to promote autophagic membrane assembly. We also demonstrated that NF2-associated mutations in Merlin attenuated the ability of Merlin to induce autophagy. And finally, we showed that Merlin knock-down was associated with enhanced cellular stress that would promote tumorigenesis.						
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Novel Role of Merlin Tumor Suppressor in Autophagy and its Implication in Treating NF2-Associated Tumors

Introduction:

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder characterized by the development of brain tumors of peripheral nervous system origin, including schwannomas, meningiomas, and ependymomas. NF2 can be inherited or sporadic, and it afflicts 1 in 25,000 people globally every year. Although tumors in NF2 patients are mostly benign, they ultimately compress neighboring nerves or increase intracranial pressure, and cause a range of devastating symptoms, including hearing loss, impaired vision or speech, and migraines. The close proximity of the NF2 tumors to critical nerves such as vestibular nerves makes complete resection difficult. In addition, due to the slow-growing nature of these tumors, chemotherapeutic intervention has been precluded as an option for NF2 treatment.

For these reasons, we endeavored to improve understanding of NF2 pathogenesis at the genetic and molecular levels. The gene causative for NF2 has been identified as the tumor suppressor Merlin, and several mutations of *Merlin* found in NF2 patients have been linked to loss of growth suppressive activity of Merlin. However, the biochemical or biological pathways that account for Merlin's tumor suppressive function have not been well understood.

A major goal of this project has been to elucidate novel functions of Merlin in autophagy, a cellular clearance system responsible for degrading old proteins or damaged organelles within cells, thus helping to mitigate the risk of tumor formation. Our preliminary data suggested that loss of Merlin leads to the attenuated autophagy as well as to enhanced hypoxia and metabolic stress, a condition known to accelerate tumor initiation. Therefore, we hypothesized that Merlin normally suppresses tumorigenesis in part by activating autophagy, and that this new role of Merlin in autophagy could be a target for therapeutic intervention for NF2-associated tumors.

To test this hypothesis, we evaluated the interaction of Merlin with autophagy-related proteins (i.e., LC3, dynein, Ulk1/Atg1). Our cell biological experiments revealed that loss of Merlin leads to attenuated autophagy. We also analyzed Merlin mutations found in NF2 patients, and found that several Merlin mutations found in NF2 patients affect the autophagy-inducing activity of Merlin. Finally, we examined the effects of metabolic stress on induction of autophagy and Merlin's role as an autophagy inducer.

We successfully confirmed the novel role of Merlin in promoting autophagy. We showed that Merlin is a part of multiprotein complex that serves as a scaffolding machinery to promote autophagic membrane assembly. We also demonstrated that NF2-associated mutations in Merlin (K79E, E270G, L46R, L64P, and V219M) attenuated the ability of Merlin to induce autophagy. And finally, we showed that Merlin knock-down was associated with enhanced cellular stress that would promote tumorigenesis.

These analyses provide new insight into the role of Merlin in autophagy and tumorigenesis, and will contribute to the development of new therapies means against NF2.

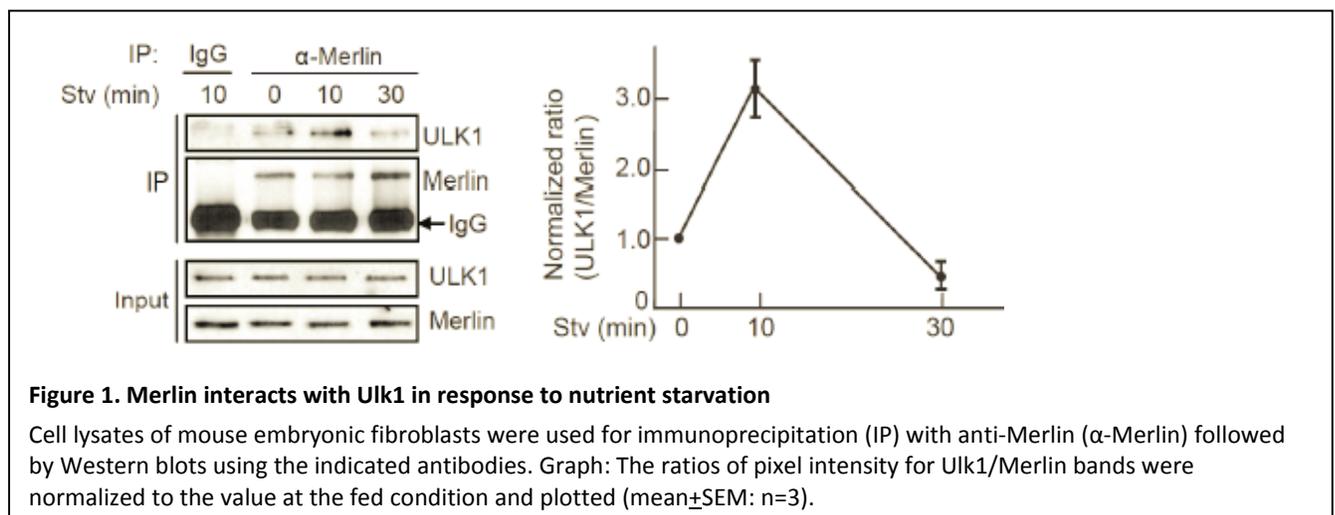
Key words: Neurofibromatosis type 2, NF2, Merlin, autophagy, tumorigenesis

Accomplishments:

Aim 1: Interactions of Merlin and autophagy-related proteins

1a. Preparation of expression constructs

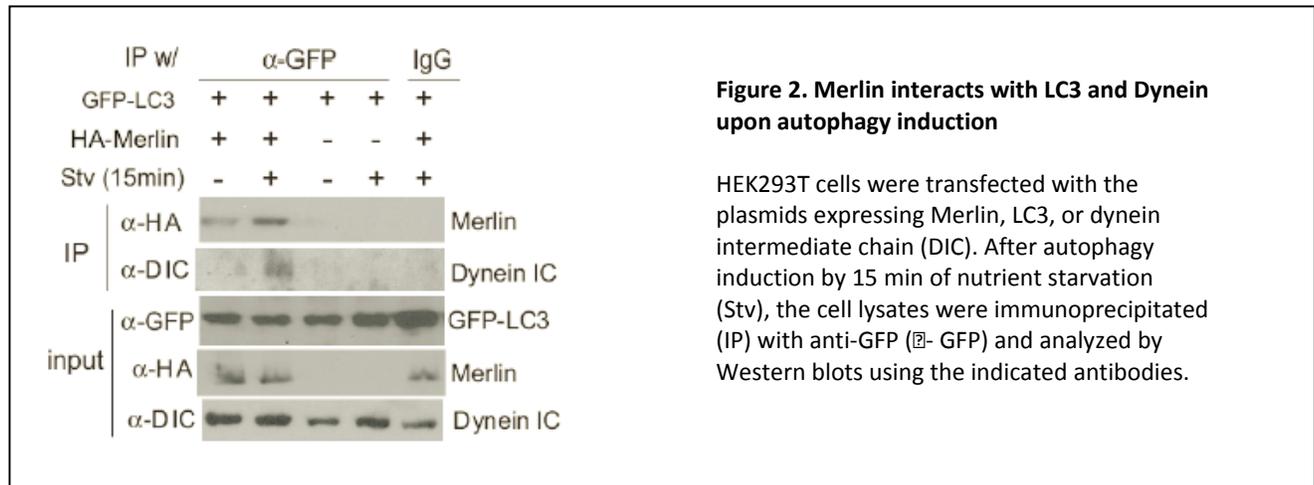
In our preliminary experiments aimed at identifying proteins that interact with Merlin tumor suppressor, we found Unc51.1/Atg1/Ulk1 as a binding partner for Merlin in the yeast two-hybrid screening. Unc51.1/Atg1/Ulk1 is an evolutionarily conserved regulator of autophagy that forms a large macromolecular complex upon autophagy induction. As a first step to study whether Merlin functions in autophagy, we characterized the Ulk1-Merlin interaction during autophagy induction. Merlin weakly bound Ulk1 under nutrient-rich conditions, and their interaction was transiently upregulated in response to nutrient starvation, showing a peak at around 10 min after autophagy induction, and rapidly downregulated by 30 min (**Figure 1**).



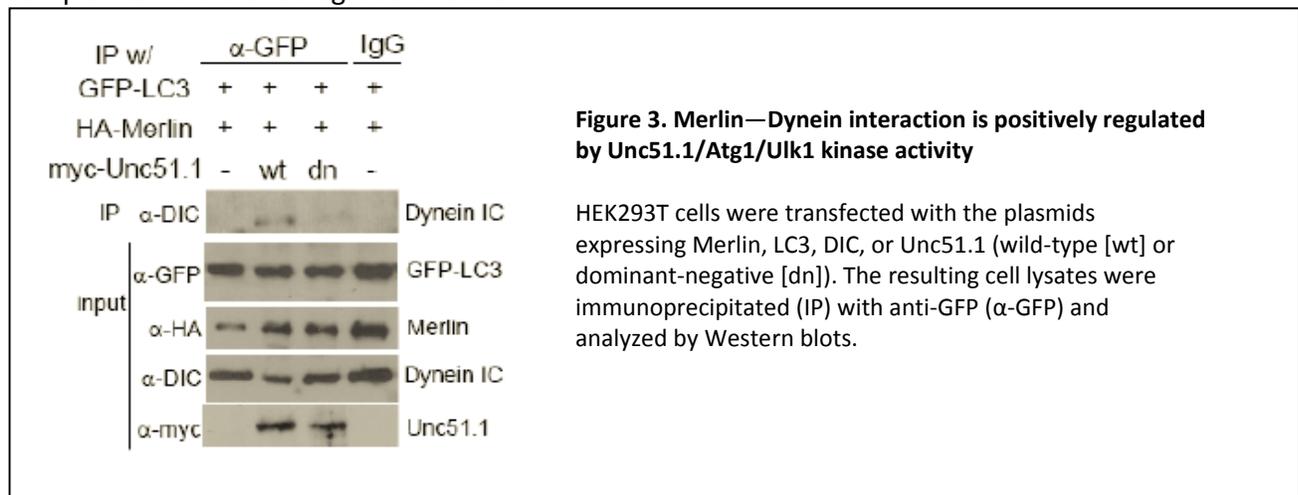
To further investigate the role of Merlin in autophagy, we analyzed the interaction of Merlin with additional autophagy-related proteins, including LC3 and dynein intermediate chain (DIC). The autophagosome marker protein LC3 is reported to bind Ulk1, and Merlin contains multiple LC3-interacting regions (LIRs). DIC is a component of dynein motor complex responsible for autophagosome trafficking, and Merlin is reported to bind dynein. We constructed the mammalian expression plasmids necessary to carry out interaction studies, including GFP-tagged LC3, myc-tagged Merlin, HA-tagged Merlin, and myc-tagged Ulk1.

1b. Transfection and immunoprecipitation experiments

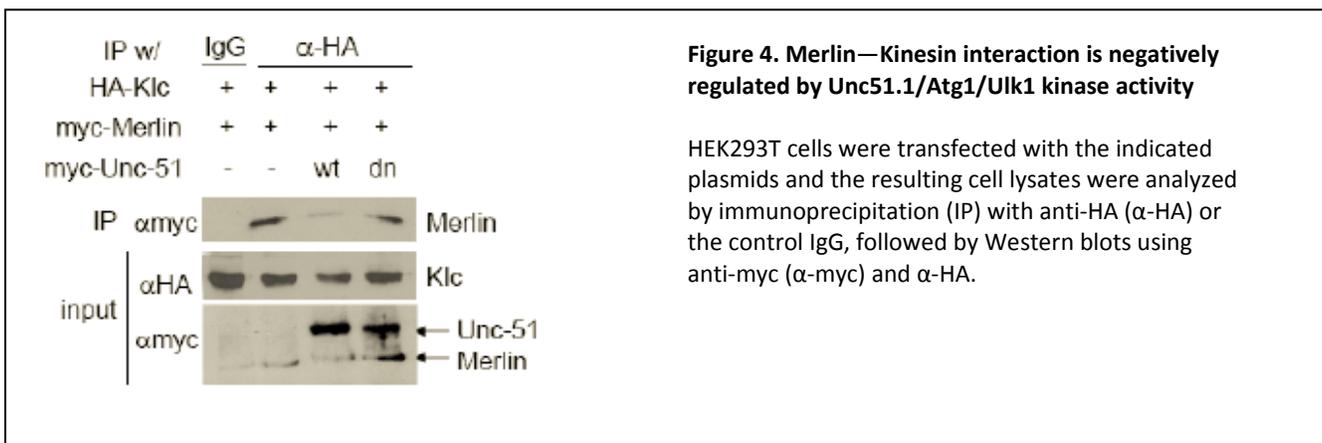
Using these reagents, we carried out immunoprecipitation experiments using HEK293T heterologous expression system. Merlin weakly bound LC3 at the fed condition, and their interaction was significantly upregulated upon autophagy induction (starvation 15min) (**Figure 2**). DIC co-precipitated with LC3 and Merlin upon autophagy induction, and the LC3—Merlin—DIC ternary complex formation was dependent on Merlin (**Figure 2**), suggesting that Merlin serves as an adaptor that links LC3 to dynein motor complex.



Because Unc51.1/Atg1/Ulk1 is a kinase critical to autophagy induction, we tested whether the kinase activity influences the formation of LC3—Merlin—DIC complex. While the wild-type kinase markedly upregulated the complex formation, the dominant-negative form inhibited the complex formation (**Figure 3**), suggesting that Unc51.1/Atg1/Ulk1 kinase activity drives the trafficking of LC3—Merlin complex toward the retrograde direction.

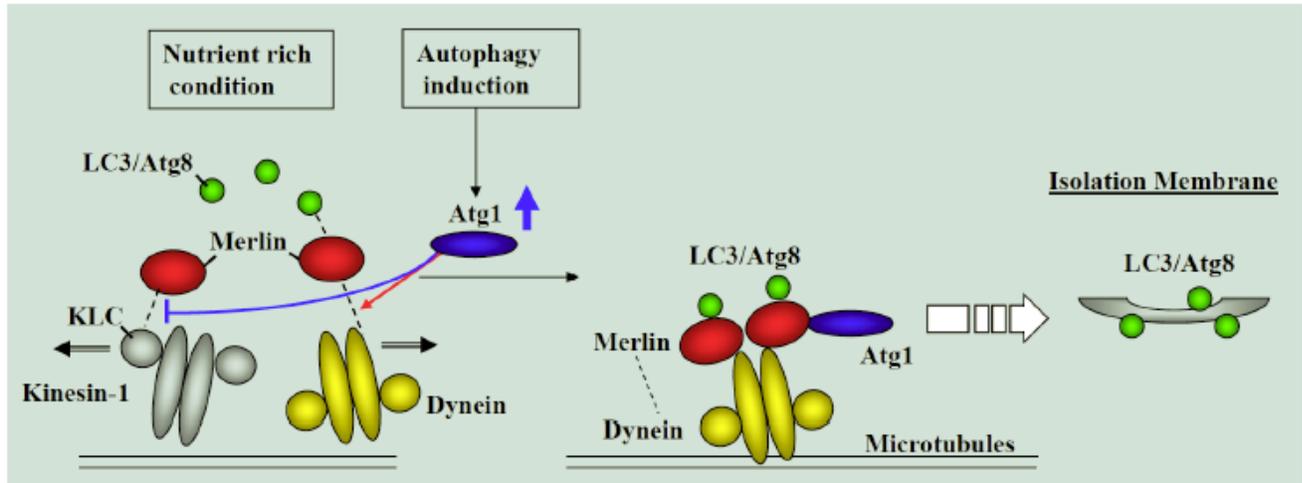


In contrast, the affinity of Merlin with kinesin light chain (Klc), a component of the kinesin anterograde motor, was inhibited by the Unc51.1/Atg1/Ulk1 kinase activity (**Figure 4**).



1c. Data analysis for 1a and 1b

Upon autophagy induction by nutrient starvation, Unc51.1/Atg1/Ulk1 kinase activity is upregulated and induces the association of Merlin and DIC, but inhibits the association of Merlin and kinesin light chain (Klc) (**Figures 1-4**). Based on these observations, we hypothesized that Merlin serves as an adaptor protein that links LC3 autophagy protein to dynein retrograde motor, thereby helping deliver LC3 towards the autophagic membranes. This model is illustrated in the diagram below:



1d. Setting up mouse crosses to prepare MEFs

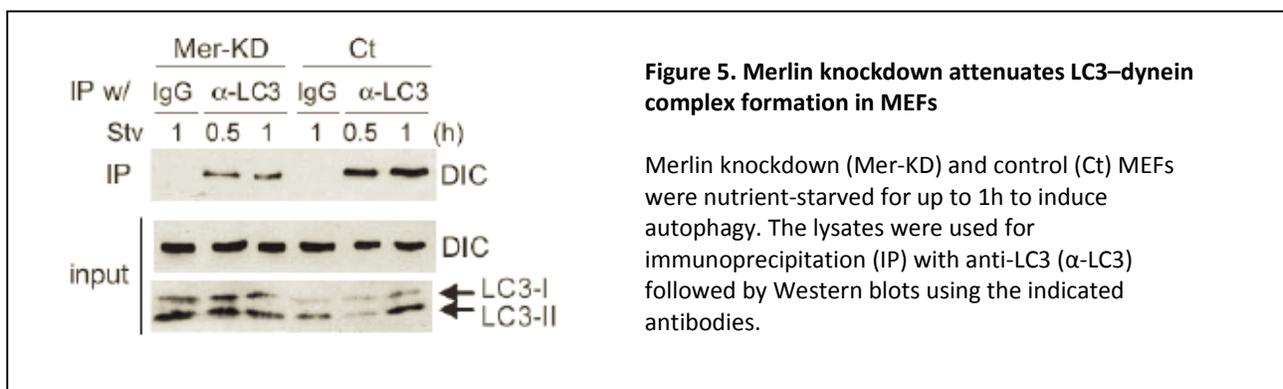
Unc51.1/Atg1/Ulk1 heterozygous mice were intercrossed and mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 14 (E14) pups of wild-type and homozygous mice.

1e. Preparation of MEFs

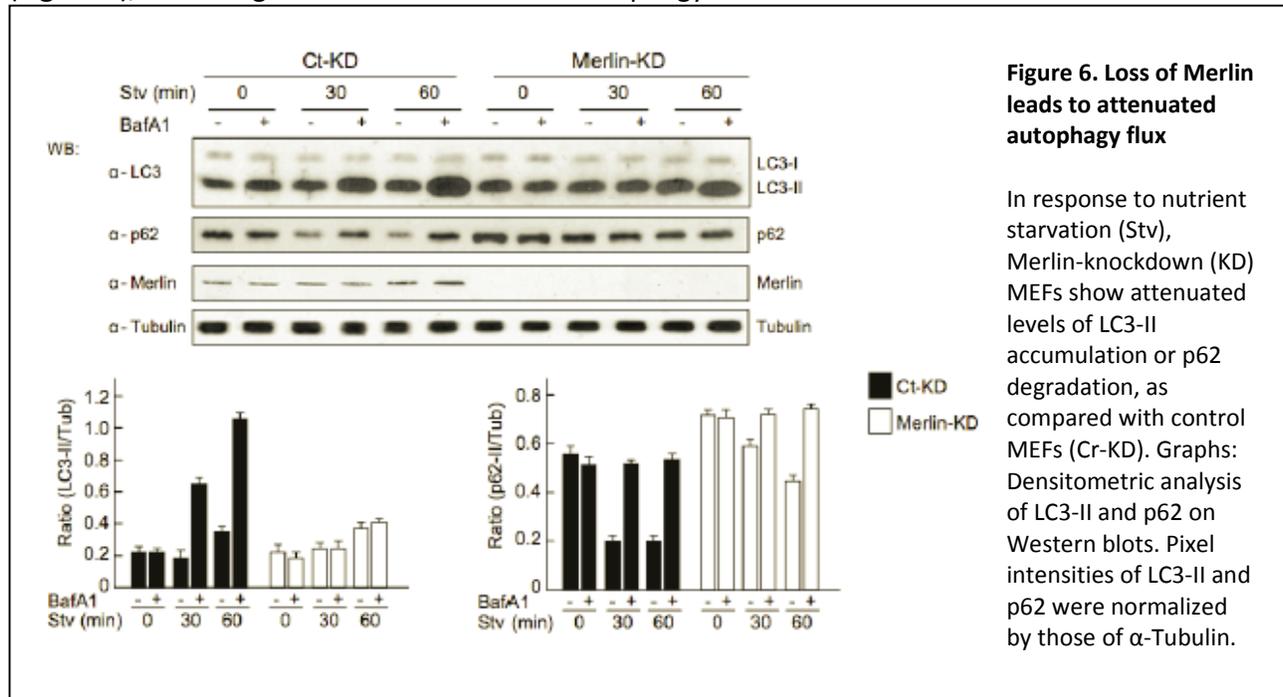
Unc51.1(-/+);GFP-LC3/+ mice crossed with *Unc51.1(-/+)* mice provided GFP-positive MEFs and GFP-negative MEFs with an expected ratio (approx. 1:1). MEFs were prepared according to standard procedures.

1f. Transfection of MEFs and interaction assays

Using MEFs prepared above, we carried out immunoprecipitation assays to confirm endogenous interaction among LC3, Merlin, and DIC. As a first step, the lentivirus constructs encoding Merlin-knockdown short-hairpin RNA (shRNA) were transfected to HEK293 cells together with the packaging plasmids to produce Merlin-knockdown lentiviruses. We then used these viruses to knockdown Merlin in MEFs. Merlin-knockdown significantly attenuated the complex formation between LC3 and DIC (**Figure 5**), suggesting that Merlin is a component of the autophagy regulatory machinery.



To prove that Merlin regulates autophagy, we measured the autophagy flux in the presence or absence of Merlin. We confirmed that Merlin knockdown MEFs showed a significant decrease in the levels of autophagy flux, as evaluated under the treatment with lysosomal protease inhibitor Bafilomycin A1 (**Figure 6**), indicating that Merlin is critical to autophagy induction



1g. Data analysis for 1f

As expected from the IP experiments using heterologous expression system (**Figures 1-4**), we showed the interaction of endogenous LC3 with DIC in MEFs. This interaction was significantly attenuated in Merlin-knockdown (KD) MEFs (**Figure 5**), consistent with the idea that Merlin serves as an adaptor linking LC3 to DIC. Moreover, Merlin-KD led to reduce levels of p62 degradation and LC3-II accumulation (**Figure 6**), suggesting that Merlin is a component of autophagy induction machinery, in which Merlin accelerates the integration of LC3 into the autophagic membranes via dynein-mediated transport.

Aim 2: Evaluation of autophagy activity of mutant Merlin

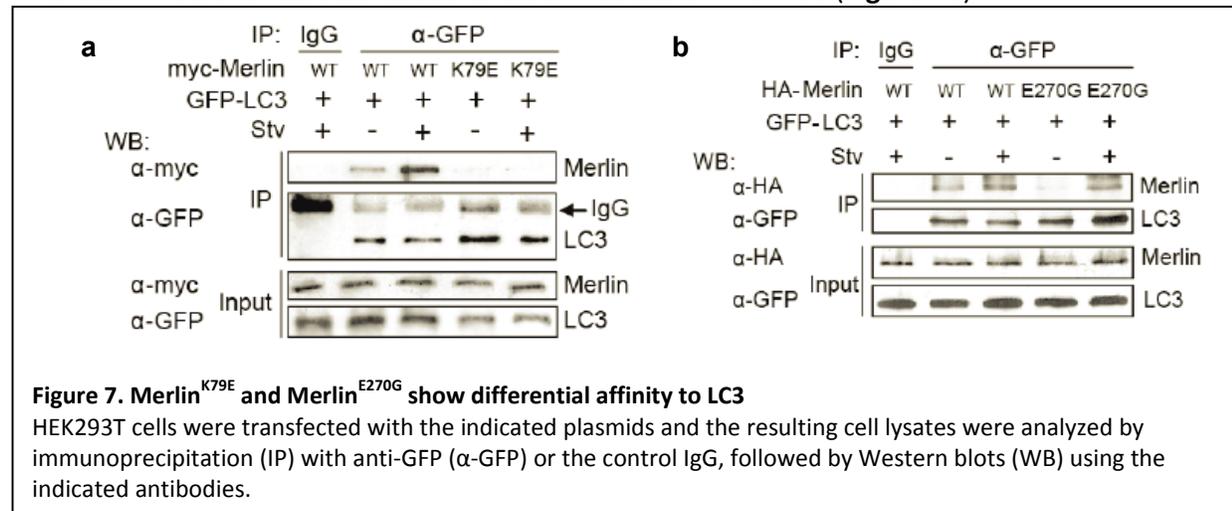
2a. Preparation of expression constructs

Studies on human NF2 revealed single amino acid mutations at >70 amino acids across the entire stretch of Merlin sequence. To determine the relevance of Merlin's role in autophagy with respect to NF2 pathogenesis, we introduced a series of representative Merlin mutations into mammalian expression constructs, and tested for their ability to induce autophagy.

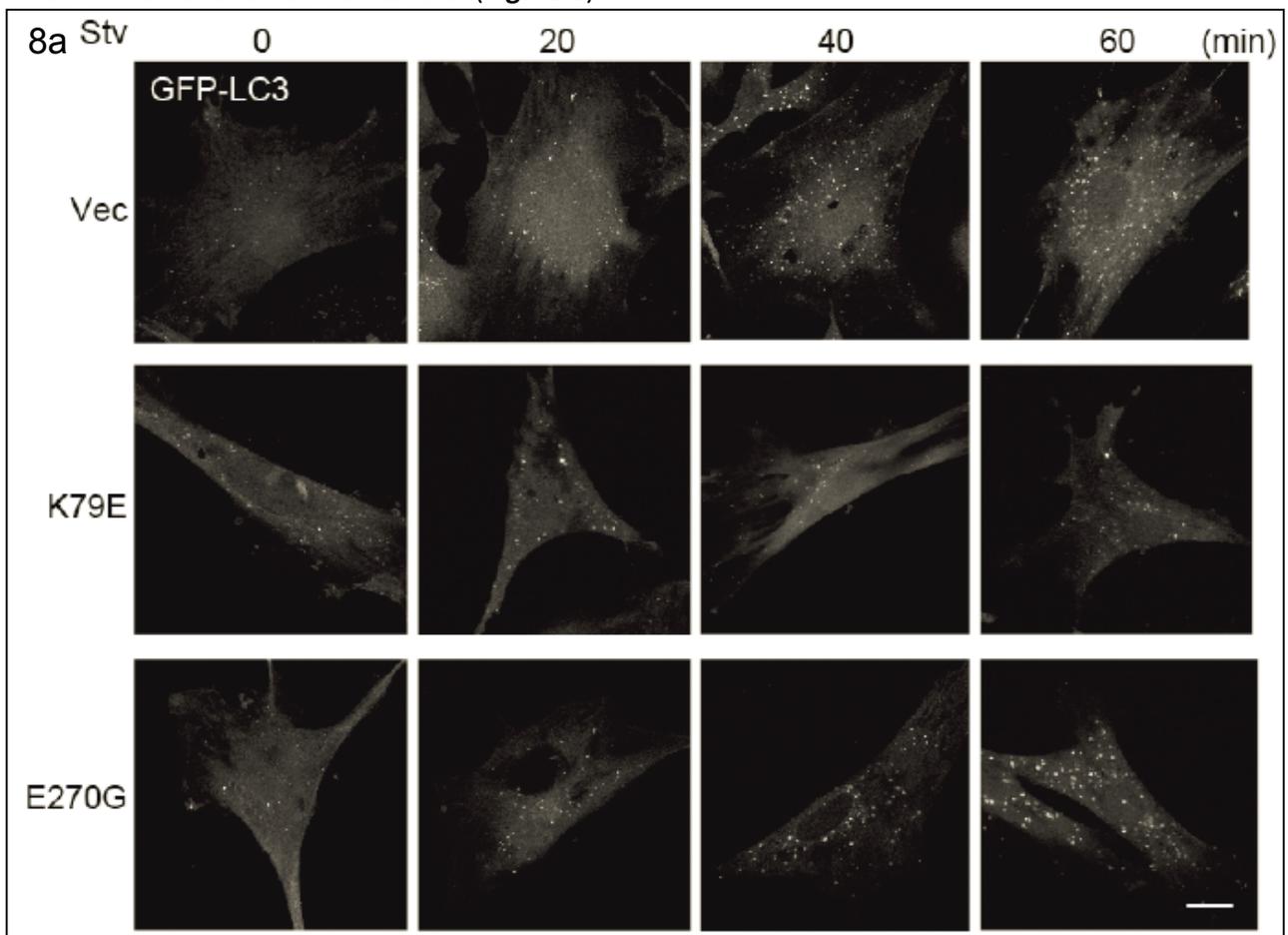
2b. Transfection and immunocytochemistry experiments

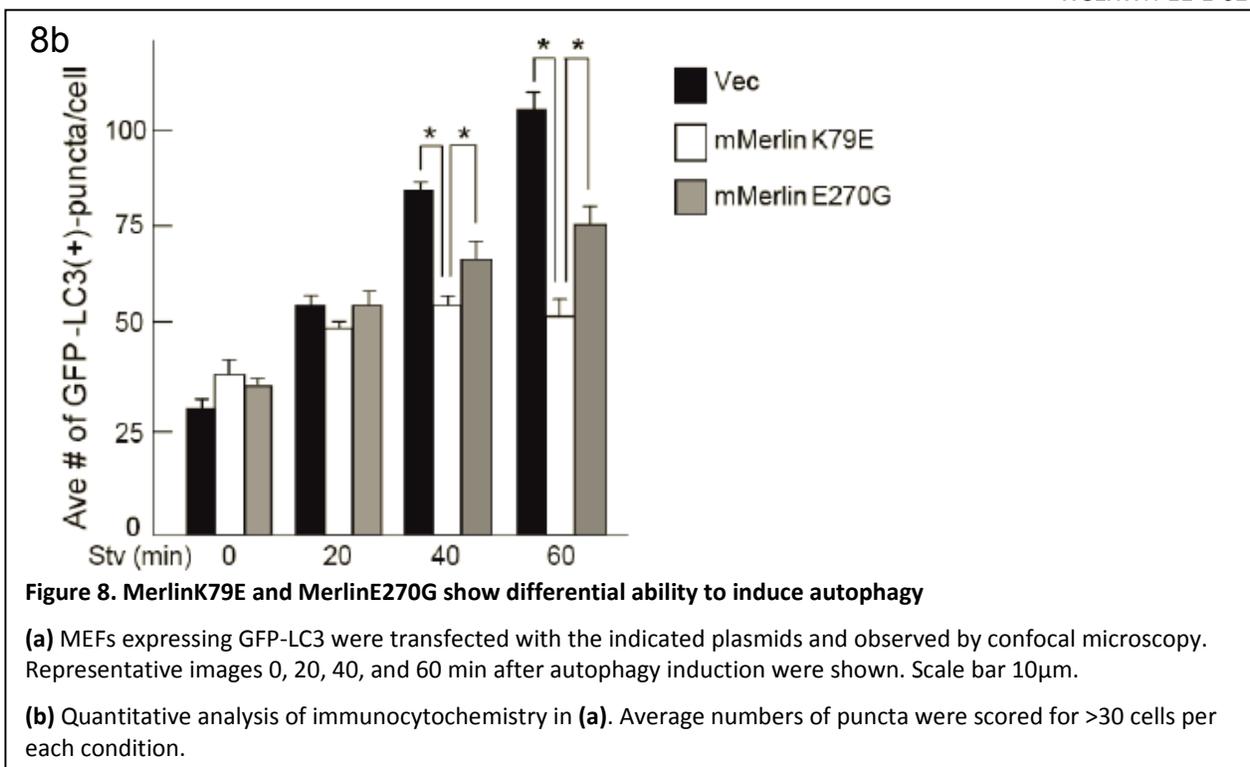
We first evaluated the Lys⁷⁹→Glu⁷⁹ (K79E) mutant and the Glu²⁷⁰→Gly²⁷⁰ (E270G) mutant in IP experiments. K79E is shown to abolish the interaction with dynein, and thus expected to impair the role of Merlin in vesicle trafficking. E270G is shown to cause hyperproliferation in cells, and thus considered

to represent tumor suppressor activity of Merlin. K79E lost the ability to interact with LC3 (**Figure 7a**), whereas E270G showed attenuated levels of interaction with LC3 (**Figure 7b**).



To evaluate the autophagy-inducing ability of these mutants, the expression constructs for Merlin K79E and E270G mutants were transfected to MEFs expressing GFP-LC3 (prepared above in Aim 1e), and numbers of GFP-LC3-positive puncta (autophagic membrane) were scored upon autophagy induction. K79E showed significantly lower levels of puncta formation than control, and E270G showed levels higher than K79E and lower than control (**Figure 8**).

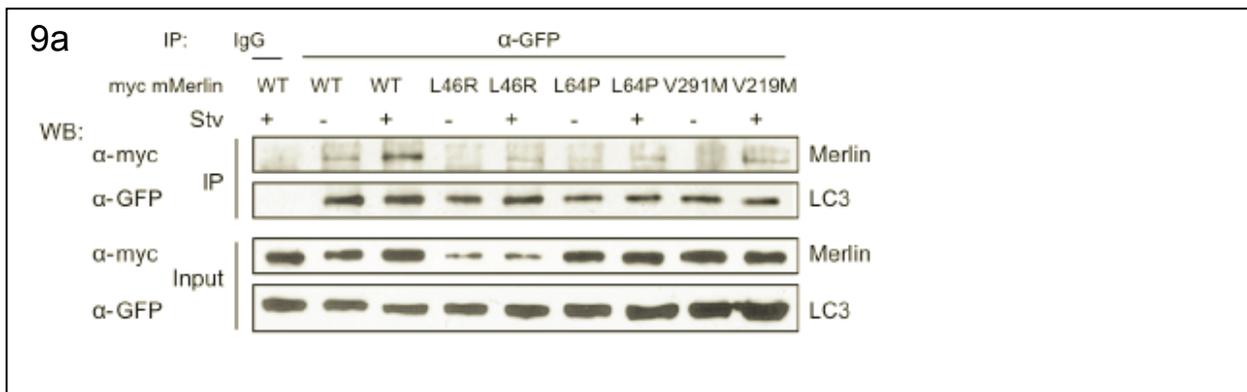




To further determine the relevance of Merlin-mediated autophagy in NF2 pathogenesis, we introduced additional mutations into Merlin expression constructs, and tested for their ability to interact with LC3 and to induce autophagy. Lys⁴⁶→Arg⁴⁶ (L46R), Lys⁶⁴→Pro⁶⁴ (L64P), and Val²¹⁹→Met²¹⁹ (V219M) mutants all showed attenuated affinity to LC3 before and after autophagy induction, when compared with wild-type Merlin (**Figure 9a**).

In addition, the autophagy-inducing abilities of these mutants were evaluated. MEFs expressing GFP-LC3 were transduced with the lentiviruses expressing each Merlin mutants (L46R, L64P, V219M), and numbers of GFP-LC3-positive puncta were scored upon autophagy induction. All these mutants showed significantly lower levels of puncta formation than Merlin WT control (**Figure 9b-d**).

In addition, the autophagy-inducing abilities of these mutants were evaluated. MEFs expressing GFP-LC3 were transduced with the lentiviruses expressing each Merlin mutants (L46R, L64P, V219M), and numbers of GFP-LC3-positive puncta were scored upon autophagy induction. All these mutants showed significantly lower levels of puncta formation than Merlin WT control (**Figure 9b-d**).



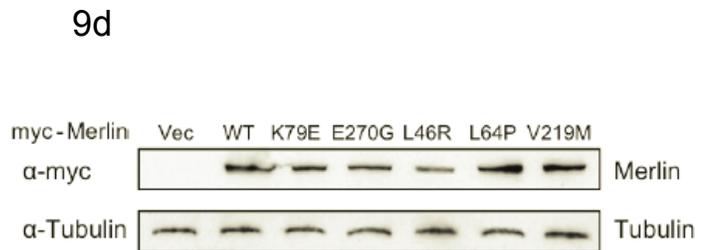
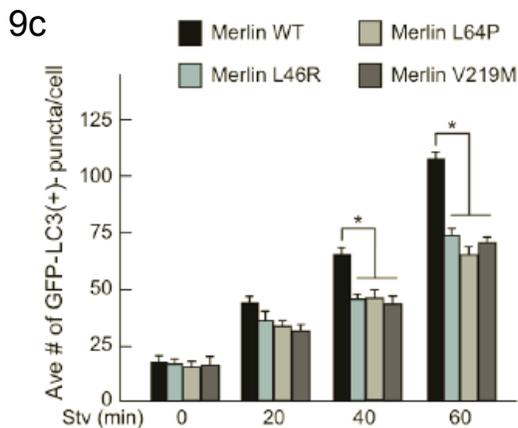
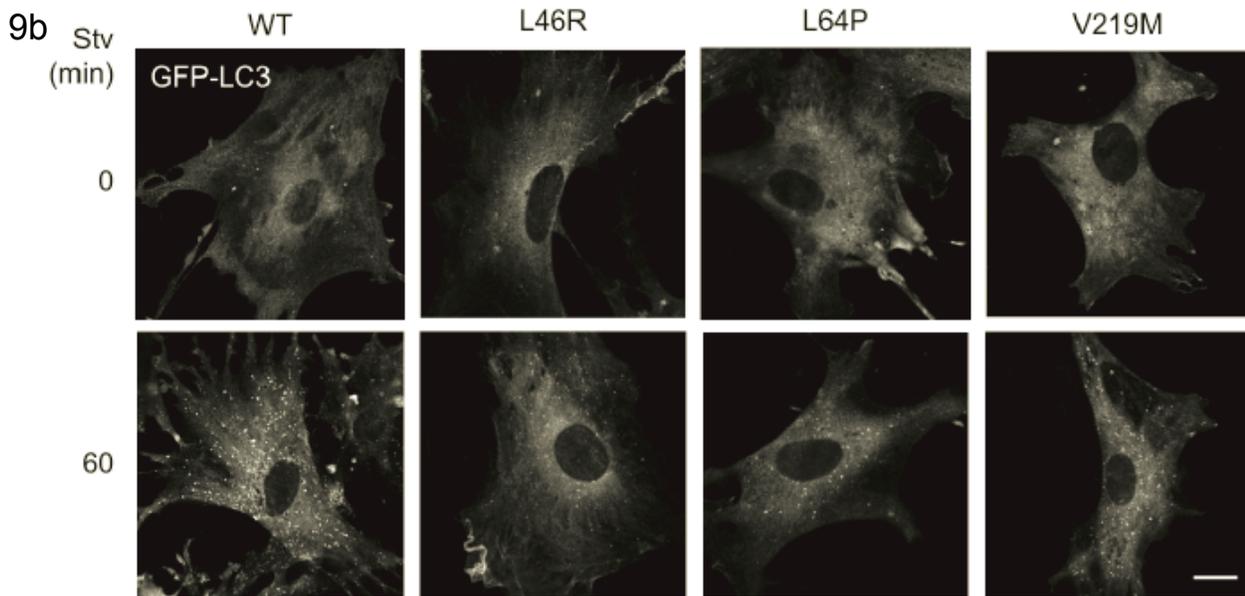


Figure 9. Merlin point mutants show weak affinity to LC3 and lower levels of autophagy induction

(a) HEK293T cells were transfected with the indicated plasmids and the resulting cell lysates were analyzed by immunoprecipitation (IP) with anti-GFP (α -GFP) or the control IgG, followed by Western blots (WB) using the indicated antibodies.

(b) MEFs expressing GFP-LC3 were infected with the lentivirus expressing the indicated Merlin mutants and observed by confocal microscopy. Representative images at the fed condition and 60 min after autophagy induction were shown. Scale bar 10 μ m.

(c) Quantitative analysis of immunocytochemistry in **(b)**. Average numbers of puncta were scored for >30 cells per each condition.

(d) MEFs that stably expressed each Merlin mutant were analyzed by WB to evaluate the level of Merlin expression.

2c. Data analysis for 2a and 2b

The results of LC3 coupling efficiency, as well as autophagy induction analyses (**Figures 7-9**), suggest that the role of Merlin in autophagy has general relevance to NF2 pathogenesis.

Aim 3: Evaluation of autophagy inhibitors in 3D culture system

3a. Preparation of MCF10A cells

We established the three-dimensional (3D) culture system using MCF10A cells. In brief, MCF10A cells were first maintained in monolayer cultures in growth media (DMEM/F12 plus 5% horse serum, 10 μ g/ml insulin, 20ng/ml EGF, 100ng/ml cholera toxin, 0.5 μ g/ml hydrocortisone, 100U/ml penicillin, and 100mg/ml streptomycin), then the 3D cultures of MCF10A were prepared on Matrigel in growth media lacking epidermal growth factor (EGF) and with reduced horse serum (2%). To induce autophagy, amino acid-free starvation media (Earle's balanced salt solution), or complete media with rapamycin (100nM) were used. Autophagy inhibitors used were: 3-methyladenine (10mM) and bafilomycin A1 (50nM).

3b. Initiate 3D cultures

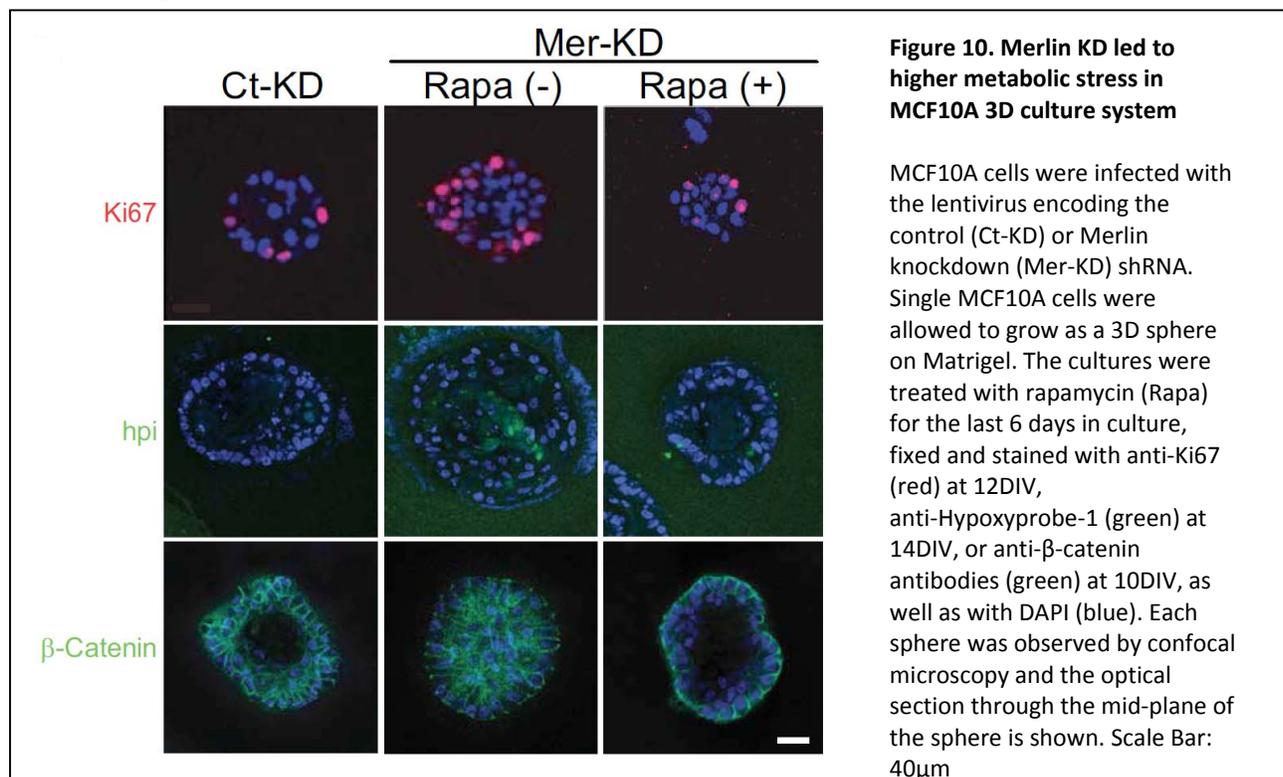
MCF10A 3D cultures were prepared by seeding 1x10⁴ MCF10A cells from the monolayer culture onto a Matrigel-coated culture chamber slide. Under this condition, single cell of MCF10A would develop into a sphere-like structure called acini within 10 to 14 days *in vitro*. Genetic manipulation, such as Merlin knockdown or Ulk1/Atg1 expression, can be accomplished by infecting MCF10A cells with a lentivirus encoding Merlin-knockdown shRNA or with a retrovirus expressing Ulk1, respectively, at the beginning of the culture when cells are at the single cell stage, and the transduced cells were selected by puromycin selection (2 μ g/ml) for 3 days.

3c. Drug treatment of MCF10A 3D cultures

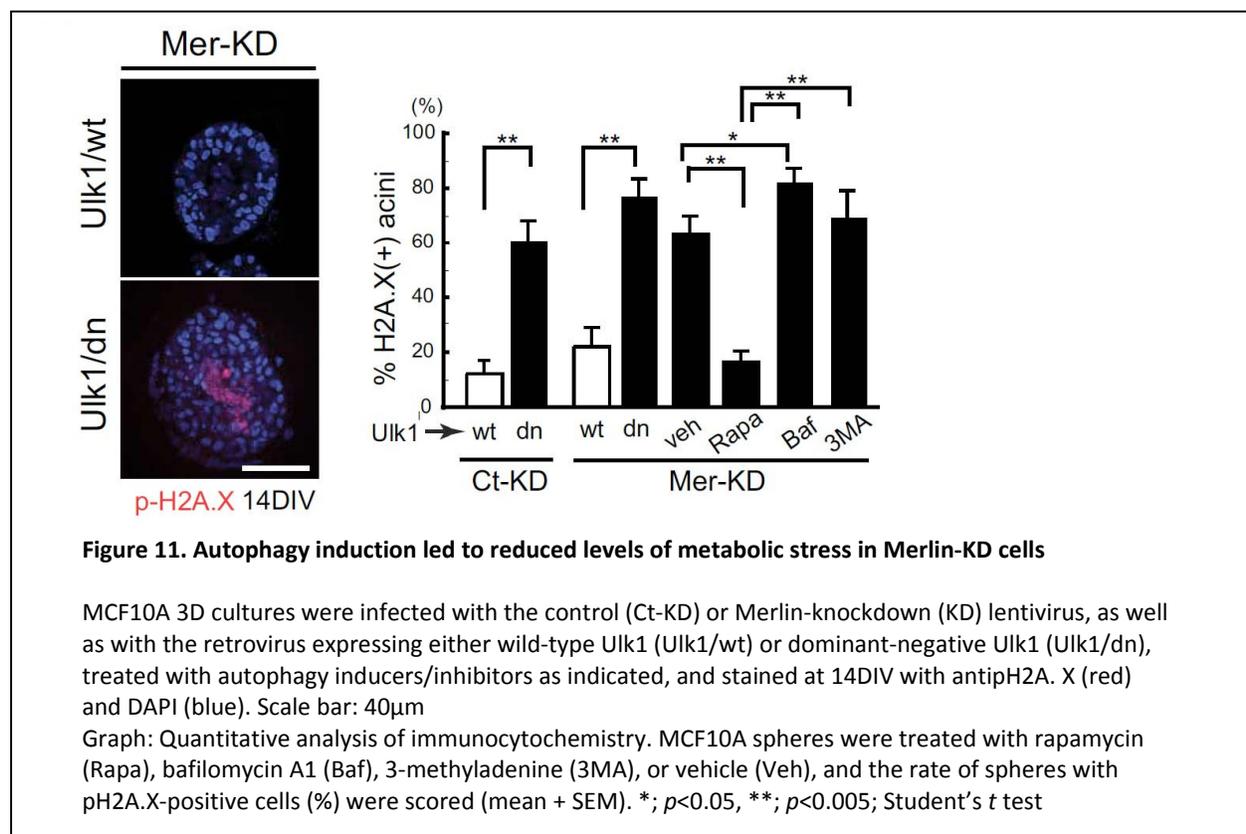
MCF10A cell 3D cultures on Matrigel were treated with a series of autophagy inducers or inhibitors for the last 6 days of the culture period, to evaluate cellular response (*i.e.*, the extent of metabolic stress as estimated by DNA damage response (phospho-histone 2A.Ser-139 [pH2A.X.] or by hypoxia marker [hpi]), cell growth (Ki-67 proliferation marker immunostaining), or a cell-shape marker (β -catenin antibody)) (**Figure 10**).

3d. Imaging analysis by microscopy

These data demonstrated that loss of Merlin resulted in higher levels of cellular proliferation (higher Ki-67), as well as higher levels of metabolic stress, as evidenced by higher hpi staining (**Figure 10**) and pH2A.X staining (**Figure 11**), as compared with control knockdown cells (Ct-KD). Rapamycin (Rapa) treatment significantly suppressed cellular proliferation and the degree of metabolic stress.



To further determine the extent to which autophagy manipulation could influence metabolic stress seen in Merlin-KD 3D cultures, autophagy was either upregulated by Ulk1/wt, or downregulated by Ulk1/dn expression, or pharmacologically inhibited by bafilomycin A1 or 3-methyladenine (3MA). Enhancing autophagy reduced the level of metabolic stress (*i.e.*, p-H2A.X), whereas inhibition of autophagy enhanced metabolic stress in 3D cultures (**Figure 11**).



3e. Data analysis for 3d

The data demonstrated that loss of Merlin resulted in higher levels of cellular proliferation (higher Ki-67), as well as higher levels of metabolic stress, as compared with control cells. Autophagy induction by rapamycin treatment or Ulk1 expression significantly suppressed the degree of metabolic stress, which is recently shown to promote tumorigenesis. Thus, the results shown in Figures 10-11 are consistent with the role of autophagy in tumor suppression, and suggest that activation of autophagy pathway could serve as a therapeutic strategy against tumors in which Merlin-mediated autophagic activity is lost or attenuated.

Conclusions:

We successfully confirmed the novel role of Merlin in promoting autophagy. We showed that Merlin is a part of multiprotein complex that serves as a scaffolding machinery to promote autophagic membrane assembly. At least one of the NF2-associated mutations (MerlinK79E) was found inhibitory to autophagy induction without showing the effect on cellular proliferation, suggesting that the role of Merlin in proliferation control vs. autophagy regulation could be separable. Additional mutations in Merlin (E270G, L46R, L64P, and V219M) also attenuated the ability of Merlin to induce autophagy. This result

demonstrates the dual role of Merlin tumor suppressor in growth suppression and autophagy induction, both of which are relevant to NF2-related tumorigenesis. Further analyses are needed to delineate the role of Merlin in autophagy.

Products :**Preparation of research papers:**

Data shown in Aim 1 through 3 have been analyzed and assembled into a paper. The manuscript has been submitted and now under revision.

Transgenic mice:

B6.129-Ulk2^{tm1Thsn}/J deposited at the Jackson Laboratory.

Key Research Accomplishments:

- Merlin promotes the accumulation of the LC3 autophagy-related protein on the autophagic membrane precursor called isolation membrane, by serving as a linker between LC3 and dynein motors.
- NF2-associated mutation MerlinK79E specifically inhibits autophagy induction without affecting its role as a growth suppressor, while the additional mutation, MerlinE270G, affects not only the growth suppressive function of Merlin but also attenuated autophagy.
- Additional NF2-associated Merlin mutations affected autophagy to a varying degree, suggesting that the role of Merlin in autophagy is relevant to NF2 pathogenesis.
- Attenuated autophagy caused by loss of Merlin led to increased levels of metabolic stress, which can be mitigated by autophagy inducer rapamycin.

Reportable Outcomes:

- June 2011. Data was presented at the poster session at Children's Tumor Foundation (CTF) annual Meeting held at Jackson Hole WY.
- June 2011. Donald Jhung Jr. obtained Ph.D. as a result of his studies supported by this award.
- October 2011. CTF-drug discovery initiative research award was obtained by Tomoda based in part on the results obtained by this award.
- June 2011-March 2014. Research associate, Ms. Yuki Hirota contributed to the majority of the results shown above.
- January 2012. Poster session describing this work presented by Ms. Hirota was selected No. 1 in the annual poster session presentation competition held at City of Hope.
- March 2012. Tomoda presented this work at the Special symposium on Autophagy; Health and Disease, held at City of Hope.
- June 2012. Tomoda presented this work at the oral session at Children's Tumor Foundation (CTF) annual Meeting held at New Orleans .
- June 2012. Dr. Akiko Sumitomo was hired and contributed to these results.
- November 2012. This work was presented by Ms. Hirota at the annual poster session held at City of Hope.
- November 2012. Tomoda presented this work at the 6th International Symposium on Autophagy held at Okinawa.

Appendices: None