Sulforaphane downregulated fatty acid synthase and inhibited microtubule-mediated mitophagy leading to apoptosis

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We previously demonstrated that sulforaphane (SFN) inhibited autophagy leading to apoptosis in human non-small cell lung cancer (NSCLC) cells, but the underlying subcellular mechanisms were unknown. Hereby, high-performance liquid chromatography-tandem mass spectrometry uncovered that SFN regulated the production of lipoproteins, and microtubule- and autophagy-associated proteins. Further, highly expressed fatty acid synthase (FASN) contributed to cancer malignancy and poor prognosis. Results showed that SFN depolymerized microtubules, downregulated FASN, and decreased its binding to α-tubulin; SFN downregulated FASN, acetyl CoA carboxylase (ACACA), and ATP citrate lyase (ACLY) via activating proteasomes and downregulating transcriptional factor SREBP1; SFN inhibited the interactions among α-tubulin and FASN, ACACA, and ACLY; SFN decreased the amount of intracellular fatty acid (FA) and mitochondrial phospholipids; and knockdown of FASN decreased mitochondrial membrane potential (ΔΨm) and increased reactive oxygen species, mitochondrial abnormality, and apoptosis.

Further, SFN downregulated mitophagy-associated proteins Bnip3 and NIX, and upregulated mitochondrial LC3 II/I. Transmission electron microscopy showed mitochondrial abnormality and accumulation of mitophagosomes in response to SFN. Combined with mitophagy inducer CCCP or autophagosome–lysosome fusion inhibitor Bafilomycin A1, we found that SFN inhibited mitophagosome–lysosome fusion leading to mitophagosome accumulation. SFN reduced the interaction between NIX and LC3 II/I, and reversed CCCP-caused FA increase. Furthermore, knockdown of α-tubulin downregulated NIX and BNIP3 production, and upregulated LC3 II/I. Besides, SFN reduced the interaction and colocalization between α-tubulin and NIX. Thus, SFN might cause apoptosis via inhibiting microtubule-mediated mitophagy. These results might give us a new insight into the mechanisms of SFN-caused apoptosis in the subcellular level.

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INTRODUCTION

We reported that sulforaphane (SFN) metabolite-induced microtubule disruption contributed to the inhibition of cell proliferation and autophagy leading to apoptosis [1, 2]. Meanwhile, SFN might cause swollen mitochondria in non-small cell lung cancer (NSCLC) [3]. In a separate study, we found that SFN might inhibit mitophagy and mitochondrial glucose metabolism via impeding the trafficking and translocation of damaged proteins into mitochondria (unpublished data). Hence, some deeper mechanisms involved in SFN-triggered mitochondrial dysfunction and metabolic abnormality might result in apoptosis. Besides, report showed that abnormal lipid metabolism might cause cell apoptosis [4]. De novo synthesis is the main pathway for cancer cells to produce fatty acids (FAs); FA synthase (FASN) is highly expressed in various cancers including lung cancer and low production of FASN in NSCLC patients have longer survival [5–7]. Study showed that downregulation of FASN production caused apoptosis in tumor cells rather than normal cells [8]. FAs are essential components of biofilm lipids and are important substrates for energy metabolism [9–11]; tumor cells require large amounts of FAs for rapid division and proliferation [12, 13].

Glucose uptake by tumor cells produces pyruvate by glycolysis, a small part of pyruvate molecules enter the mitochondria to undergo oxidative decarboxylation to form citric acids. Citric acids were transported out of mitochondria and catalyzed by ATP citrate lyase (ACLY) to release acetyl CoA. Acetyl CoA carboxylase (ACACA) catalyzes acetyl CoA to form malonyl CoA. In the presence of nicotinamide adenine dinucleotide phosphate, acetyl CoA interacts with malonyl CoA to synthesize FAs by FASN [14]. FA metabolites might act as signal molecules involved in tumor growth or participate in protein modification after translation [8]. Therefore, the interference of FA synthesis by inhibiting FA synthesis-related molecules might help us establish an effective anti-cancer therapy.

Studies showed that cruciferous plant-derived SFN inhibited lipid production and induced apoptosis in prostate cancer cells [13, 15]. SFN induced apoptosis of NSCLC cells in a concentration-dependent manner at 20 μM concentration [3]. Just recently, we reported that the combination of SFN metabolites and paclitaxel reduced the dosage and restored sensitivity of paclitaxel-resistant cells to paclitaxel [16]. We also

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HPLC-MS/MS analysis showed that SFN affected the production of lipoproteins, membrane proteins, and autophagy-, apoptosis-, and microtubule-associated proteins.

| Lipid metabolism | Membrane | Microtubule | Apoptosis | Autophagy |
|------------------|----------|-------------|-----------|-----------|
| **Downregulated proteins** | | | | |
| Fatty acid synthase | Isoform 3 of calcium-binding mitochondrial carrier protein | Tubulin β-4B chain | Pleckstrin homology-like domain family A member 3 | Phosphatidylinositol 3-kinase catalytic subunit type 3 |
| Isoform 3 of 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase β-4 | Oxysterol-binding protein-related protein 3 | Tubulin α-4A chain | Protein JTB | Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β-isofom |
| Isoform 3 of cytosolic phospholipase A2 epsilon | Ras-related protein | Tubulin α-1C chain | Tensin-4 | Ubiquitin-like modifier-activating enzyme ATG7 |
| Acyl-protein thioesterase 2 | Peroxisomal membrane protein | Isoform 3 of echinoderm microtubule-associated protein-like 2 | Isoform 2 of insulin-like growth factor-binding protein 3 | RB1-inducible coiled-coil protein 1 |
| 15-Hydroxyprostaglandin dehydrogenase [NAD+] | Phosphatidylinositol glycan anchor biosynthesis class U protein | β5-Tubulin | | Isoform 5 of autophagy-related protein 13 |
| **Upregulated proteins** | | | | |
| Apolipoprotein A-I | Isoform 2 of ATP-binding cassette subfamily B member 7 | Dynein heavy chain 2 | BAG family molecular chaperone regulator 3 | Ubiquitin-like protein ATG12 |
| Apolipoprotein C-III | Essential MCU regulator | Kinesin-like protein KIF23 | Cytoskeleton-associated protein 2 | Isoform 2 of autophagy-related protein 2 homolog A |
| Serine incorporator 1 | ATP synthase subunit e | Isoform 2 of targeting protein for Xklp2 | Tumor necrosis factor receptor superfamily member 10D |
| MICOS complex subunit | Nucleolar and spindle-associated protein 1 | Cytochrome c | | Programmed cell death protein 7 |
found that SFN metabolites induced apoptosis by inhibiting microtubule-mediated autophagy in NSCLC [1]. Microtubules are the dimers formed by α-tubulin, β-tubulin, and a small part of microtubule-binding proteins, such as microtubule-associated protein 1 light chain 3 (LC3 II/I) and stathmin-1 [17]. However, it was not clear whether SFN-caused microtubule disruption contributed to inhibition of FA synthesis and mitophagy. In particular, whether the inhibition of mitophagy regulated FA production leading to apoptosis was unknown.

Bafilomycin A1 (Baf-A1) is an autophagy inhibitor that blocks the fusion of autophagosome to lysosome. We previously reported that, combined with Baf-A1, SFN metabolites inhibited the fusion of autophagosome to lysosome and caused the accumulated autophagy flux LC3 II/I leading to apoptosis [1]. LC3 II/I located on the autophagic membrane and were autophagy marker proteins [18]. Mitophagy helps cells to remove damaged mitochondria and maintain cellular proteostasis [19, 20]. Mitophagy is mainly mediated by the PARKIN/Parkin, BNIPI/NIX, and FUNDC1 signaling pathways. NIX is a NIP3 protein on mitochondria in the Bcl-2 family. BNIPI is a member of the Bcl-2 protein family and belongs to the BH3 subfamily containing only the BH3 domain [21, 22]. The damaged mitochondria are surrounded by a bilar membrane to form mitophagosomes. NIX on the outer membrane of mitochondria recruits LC3 II/I to form autophagosomes enveloping damaged mitochondria; the phagocytosis of damaged mitochondria maintains the homeostasis of cells [23, 24]. Consequently, SFN might inhibit the fusion of mitophagosomes to lysosomes and the recruitment of LC3 II/I to NIX [25].

Taken together, we hypothesized that SFN inhibited microtubule-mediated FA synthesis disrupting mitochondrial membrane structures and inhibited microtubule-mediated mitophagy leading to apoptosis. These studies might help us understand the deeper mechanisms that SFN produced in the subcellular levels and establish efficient anti-cancer therapies.

RESULTS
SFN-caused apoptosis might result from the downregulation of FA synthesis-associated proteins and microtubule-associated proteins
These results by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis showed that...
SFN affected the production of lipoproteins, membrane proteins, and autophagy-, apoptosis- and microtubule-associated proteins (Table 1). Further, the rates of apoptosis were increased and cleaved caspase-3 level was increased after 24 h treatment with SFN (Fig. 1A, B). Cell apoptotic features, such as chromatin condensation, apoptotic bodies, and vacuolization were viewed by a transmission electron microscope (TEM) (Fig. 1C). Tissue microarray analysis showed a positive correlation of FASN level to the malignancy of NSCLC (Fig. 1D). FASN level is positively correlated to the malignant grades of tumor cells in both lung squamous cell carcinoma and lung adenocarcinoma (Fig. 1E). The correlation of FASN expression to pathological grading of NSCLC was analyzed by calculating the H-score (Table 2). The level of FASN in tumor tissues is higher than that in adjacent tissues (Fig. 1F). These results suggested that SFN-caused apoptosis might result from the downregulation of FA synthesis-associated proteins and the dysfunction of microtubule- and autophagy-associated proteins, and membrane proteins.

SFN inhibited the polymerization of microtubules
Cells were treated with different concentrations of SFN for 24 h. Results showed that SFN significantly downregulated α-tubulin in a concentration-dependent manner (Fig. 2A). Meanwhile, β-tubulin was also downregulated in a concentration-dependent manner (Fig. 2B). Microtubule polymerization assays showed that the amounts of both soluble and insoluble α-tubulin or β-tubulin were lower vs. control, indicating that the SFN depolymerized the microtubules (Fig. 2C, D). Immunoﬂuorescence staining showed that SFN disrupted microtubules and reduced the colocalization of α-tubulin to β-tubulin. Scale bar: AS-49: 75 µm, SK-1: 25 µm. Data were shown as means ± SEM (n ≥ 3). **P ≤ 0.001; ***P ≤ 0.0001.

SFN inhibited microtubule-mediated FA synthesis via activating 26S proteasome
After treatment with gradient concentrations of SFN for 24 h, the results showed that SREBP1, FASN, ACACA, and ACLY were
downregulated in a concentration-dependent manner (Fig. 3A). Combined with proteasome inhibitor PS-341, the results show that SFN degraded SREBP1, FASN, ACACA, and ACLY via activating 26S proteasome (Fig. 3B). After knockdown of SREBP1 production, the FA-related proteins ACACA, FASN, and ACLY were downregulated (Fig. 3C). These results showed that SFN degraded the key enzymes in FA synthesis through two pathways, the 26S-proteasomal pathway and the downregulation of SREBP1, as a transcriptional factor for FASN production. Correlation analysis showed that level of SREBP1 had a correlation to FASN level and the level of either ACACA or ACLY, or FASN was closely correlated to FA synthesis (Fig. 3D). Moreover, we found that SFN inhibited the interaction between α-tubulin and FASN, ACACA, and ACLY (Fig. 3E); meanwhile, SFN lowered the colocalization of FASN, ACACA, and ACLY to α-tubulin (Fig. 3F). After knockdown of β-tubulin production, the FA-related proteins ACACA, FASN, and ACLY was downregulated (Fig. 3G). These results showed that disrupted microtubule led to decrease of expression level of FA-related proteins. The levels of FAs and mitochondrial phospholipids were significantly reduced in response to SFN (Fig. 3H, I). Therefore, SFN might lower the production of mitochondrial phospholipids by inhibiting microtubule-mediated FA synthesis leading to the dysfunction of mitochondrial membranes.

SFN-triggered downregulation of FASN level contributed to apoptosis

The FASN level was successfully knocked down via small interfering RNA (siRNA) interference leading to the elevated intracellular reactive oxygen species (ROS) level and downregulation of mitochondrial membrane potential (ΔΨm) (Fig. 4A–C). Abnormal cell membrane structures, swollen mitochondria, and apoptotic features were viewed (Fig. 4D). Knockdown of FASN production induced cell apoptosis (Fig. 4E). The levels of FA and mitochondrial phospholipid were significantly reduced in the FASN siRNA group vs. control (Fig. 4F, G). After knockdown of FASN production, the microtubule-associated protein LC3 II/I was upregulated (Fig. 4H). These results indicated that inhibition of FASN production might reduce cellular FA content, thereby reducing intracellular phospholipid level, leading to mitochondrial membrane instability and apoptosis.

SFN inhibited microtubule-mediated mitophagy and the fusion of mitophagosomes to lysosomes

We found that mitophagy-associated protein BNIP3 localized to mitochondria and SFN downregulated BNIP3 level (Fig. 5A), and the mitochondrial mitophagy-associated protein NIX was expressed in both the mitochondria and cytoplasm, and the expressions were inhibited by SFN in a concentration gradient (Fig. 5B, C). The accumulated microtubule-associated protein LC3 II by SFN was mainly present in mitochondria and SFN treatment had no significant effect on LC3 II/I in cytoplasm (Fig. 5D, E). Mitophagosomes in the cells were viewed after treated with SFN, whereas autolysosome was rarely visible (Fig. 5F). After co-treated with the mitophagy inducer carbonylcyanide 3-chlorophenylhydrazone (CCCP), SFN reversed LC3 II/I downregulation (Fig. 5G, H). After co-treated with Baf-A1, SFN did not make a significant change in LC3 II/I level (Fig. 5I, J). These results indicated that SFN inhibited mitophagy by hindering the fusion of mitophagosomes to lysosomes. Coimmunoprecipitation showed that SFN reduced the interaction between NIX and LC3 II/I in the cytoplasm and mitochondria, and inhibited the initiation of mitophagy (Fig. 5K, L). These results showed that SFN inhibited mitophagy by lowering the expression of mitophagy-associated proteins and the interaction between microtubule-associated protein LC3 II/I and mitophagy-associated proteins inhibiting the degradation of mitophagosomes.

SFN induced apoptosis by inhibiting microtubule-mediated FA synthesis and mitophagy

After the cells were treated with the mitophagy inducer CCCP, FA levels were increased. Combined with SFN, the increased FA

| Variable       | Lung squamous carcinoma | P-value | Lung adenocarcinoma | P-value |
|----------------|-------------------------|---------|---------------------|---------|
|                | All patients | Low | High |                | All patients | Low | High |                |
| Gender         | 0.458        |      |      |                | 0.567        |      |      |                |
| Male           | 71           | 19  | 52   |                | 39           | 10  | 29   |                |
| Female         | 4            | 2   | 2    |                | 35           | 7   | 28   |                |
| Age (years)    | 0.893        |      |      |                | 0.136        |      |      |                |
| >60            | 40           | 12  | 28   |                | 41           | 12  | 29   |                |
| ≤60            | 35           | 10  | 25   |                | 34           | 5   | 29   |                |
| Differentiation| 0.001*       |      |      |                | 0.003*       |      |      |                |
| I              | 6            | 5   | 1    |                | 1            | 14  | 7    |                |
| II             | 44           | 14  | 30   |                | 42           | 10  | 32   |                |
| III            | 25           | 2   | 23   |                | 19           | 0   | 19   |                |
| Staging        | 0.144        |      |      |                | 0.838        |      |      |                |
| IA             | 9            | 1   | 8    |                | 14           | 4   | 10   |                |
| IB             | 14           | 4   | 10   |                | 23           | 5   | 18   |                |
| IIA            | 13           | 7   | 6    |                | 14           | 2   | 12   |                |
| IIB            | 10           | 3   | 7    |                | 4            | 1   | 3    |                |
| IIIA           | 22           | 6   | 16   |                | 11           | 4   | 7    |                |
| IIIB           | 2            | 0   | 5    |                | 3            | 0   | 3    |                |
| IV             | 5            | 0   | 5    |                | 2            | 0   | 2    |                |

* *p < 0.05 was defined as statistically significant.

Table 2. Correlation of FASN expression to clinicopathological characteristics of lung cancer patients.

| Variable | 0.458 | 0.567 |
|----------|-------|-------|
| Gender   | 0.458 | 0.567 |
| Male     | 0.458 | 0.567 |
| Female   | 0.458 | 0.567 |
| Age (years) | 0.893 | 0.136 |
| >60 | 0.893 | 0.136 |
| ≤60 | 0.893 | 0.136 |
| Differentiation | 0.001* | 0.003* |
| I | 0.001* | 0.003* |
| II | 0.001* | 0.003* |
| III | 0.001* | 0.003* |
| Staging | 0.001* | 0.003* |
| IA | 0.001* | 0.003* |
| IB | 0.001* | 0.003* |
| IIA | 0.001* | 0.003* |
| IIB | 0.001* | 0.003* |
| IIIA | 0.001* | 0.003* |
| IIIB | 0.001* | 0.003* |
| IV | 0.001* | 0.003* |
levels were reversed (Fig. 6A). The analysis via Phospholipid Kits showed the consistent results (Fig. 6B). These indicated that SFN-inhibited mitophagy contributed the decrease of FA. Reversely, the decrease might inhibit mitophagy. After knocking down of α-tubulin, mitophagy markers BNIP3 and NIX levels were downregulated (Fig. 6C, D). Meanwhile, LC3 II/I levels were upregulated (Fig. 6E). These indicated that SFN inhibited the binding of mitophagosome to lysosome by decreasing α-tubulin. Coimmunoprecipitation showed that the interaction between α-tubulin and NIX was downregulated in both the mitochondria and cytoplasm (Fig. 6F, G). Confocal microscopy observation showed that SFN inhibited the localization of α-tubulin to NIX (Fig. 6H). These results indicated that SFN inhibited mitophagy regulating FA level via a microtubule-mediated way. Flow cytometry showed that cell apoptosis rates were decreased after co-treated with CCCP and the apoptosis rates were increased after co-treated with Baf-A1 (Fig. 6I, J). Taken together, these data demonstrated that SFN inhibited microtubule-mediated FA synthesis and mitophagy leading to apoptosis (Fig. 6K).
DISCUSSION
Here we found that SFN inhibited microtubule-mediated FA synthesis and mitophagy resulting in decreased FA, mitochondrial dysfunction, and apoptosis. Consequently, damaged mitochondria had difficulty in maintaining their stability and protein homeostasis. These results will help us understand the underlying mechanisms that SFN triggered apoptosis in the subcellular level. HPLC-MS/MS analysis showed that FASN, ACACA, α-tubulin, and β-tubulin were the target molecules, which SFN initiated anticancer signaling to downregulate. Previous studies showed that SFN might bind to α-tubulin, β-tubulin to degrade α-tubulin, and β-tubulin causing apoptosis [26]. α-tubulin is the main component...
of microtubules and might bind tightly to β-tubulin to form microtubule dimers [27]. Microtubule acts as the cytoskeleton to support a variety of proteins for transport and scaffolding [28, 29]. Here we further uncovered that SFN inhibited the formation of dimers consisting of α-tubulin and β-tubulin, resulting in microtubule depolymerization. These results indicated that SFN-triggered microtubule disruption played a role in FA synthesis and metabolism.

Interestingly, SFN treatment caused a decrease in total free FA and mitochondrial phospholipids. Also, the levels of a few lipid enzymes involved in FA synthesis were also decreased upon SFN treatment in cultured cancer cells. FA is critical for energy metabolism and is the fundamental component of all cell membrane [30]. FASN is a key enzyme involved in the synthesis of FA essential for the de novo synthesis of FA in tumor cells [10, 31]. Therefore, it is helpful to establish a new anti-cancer therapy via targeting FA synthesis and metabolism signaling. TVB-2640 is a clinically effective FASN inhibitor, which works greatly in the treatment of patients with NSCLC [10]. However, most FASN inhibitors are not used for clinical study due to poor stability and insufficient bioavailability such as cerulenin and orlistat. It was expected that SFN would have an excellent oral bioavailability, because SFN exerted a synergistically anti-cancer effect in combination with paclitaxel reducing the dosage of both agents [32, 33]. Moreover, NSCLC patients with higher level of FASN have lower survival. By immunohistochemical staining in microarray tissues, we found that FASN was highly expressed in tumors and was associated with malignant grades of tumors. As the most critical transcription factor regulating lipid synthesis, SREBP1 is critical for the de novo synthesis of FA [34]. It was reported that ACLY, ACACA, and FASN levels were positively correlated to their morphology, function, and quantity to accommodate the metabolic needs of cells. Mitophagy might effectively remove damaged mitochondria by PINK/Parkin, BNIP3/LNIX, and FUNDC1 [41, 42]. Our results showed that the total free FA and mitochondrial phospholipids were increased after combined with CCCP. This suggested that SFN decreased mitochondrial phospholipid levels, thereby disrupting the mitochondrial membrane. Destruction of integrity of the mitochondrial membrane might be one of the reasons to inhibit the formation of autophagosomes and mitophagy [43]. On the contrary, decreased FA levels might inhibit mitophagy leading to apoptosis.

In NSCLC, we reported that SFN metabolites inhibited microtubule-mediated autophagy leading to apoptosis [44]. Here we found more profound mechanisms in the subcellular level. SFN mainly inhibited microtubule-mediated FA production and microtubule-regulated mitophagy affecting energy metabolism. Mitochondria acted as dynamic organelles that constantly modulated their morphology, function, and quantity to accommodate the metabolic needs of cells. Mitophagy might effectively remove damaged mitochondria by PINK/Parkin, BNIP3/LNIX, and FUNDC1 pathways [45]. The accumulation of dysfunctional mitochondria was associated with many human diseases including cancer [46]. It was associated that BNIP3L/NIX regulated the removal of the mitochondria. This mitophagy receptor bound to the mitochondria and phagocytic membrane protein LC3 II/I, and promoted mitochondrial encapsulation in autophagosomes, then it was delivered to lysosomes for degradation [46]. Both NIX and BNIP3 localized to the outer mitochondrial membrane by way of a C-terminal transmembrane domain [47]. Also, both of them directly interacted with the autophagosome membrane protein LC3 [48]. Here, we found that SFN inhibited mitophagy via downregulating BNIP3 and NIX. Further, our study showed that SFN inhibited the interaction between NIX and LC3. These results demonstrated that SFN inhibited the formation of mitophagosomes. The interaction between α-tubulin and NIX was determined after treated with SFN. After knockdown of α-tubulin, the levels of BNIP3 and NIX were downregulated, indicating that SFN induced microtubule disruption resulting in inhibition of mitophagy.

Although autophagy was discovered for more than 50 years, the relationship between mitophagy and cell death remained elusive [49]. It was reported that autophagy protected cells by maintaining energy homeostasis and nutrient requirements under starvation conditions. In tumor cells, autophagy might contribute to the undesired survival of tumor cells [50, 51]. By co-treatment with mitophagy inducer CCCP, we demonstrated that SFN decreased FA levels inhibiting mitophagy, leading to apoptosis in NSCLC cells. These gave us some new hints that SFN might disturb microtubule-mediated synthesis and mitophagy. Meanwhile, SFN might interfere with the cross-talks between FA metabolism and mitophagy.
Taken together, we uncovered a brand new mechanism that SFN induced apoptosis via inhibiting microtubule-mediated FA synthesis and mitophagy. These results might help us to establish new anti-cancer therapies via targeting lipid signaling and mitophagy-associated molecules.

MATERIALS AND METHODS

Antibodies and reagents
Anti-α-tubulin (YM3035) and anti-β-tubulin (YM3139) were purchased from ImmunoWay Biotechnology (TX, USA); anti-FASN (SC-48357), anti-NIX (SC-166332), and anti-SREBP1 (SC-13551) were purchased from Santa Cruz Biotech (TX, USA); anti-BNIP3 (D121876) and anti-ACLY (D221957) were...
Western blotting was used to analyze BNIP3 level in the mitochondria. After SFN was used at a designated concentration for 24 h, the mitochondrial isolation kit was used to separate the cytoplasm and mitochondrial protein lysates. Western blotting was used to analyze NIX level in the cytoplasm and mitochondria. Western blotting was used to analyze LC3 II/I accumulation in the cytoplasm and mitochondria (E). Cells were treated with or without 20 μM SFN for 24 h. The images via TEM showed the mitophagosomes; double red arrows marked mitophagosomes; black arrows marked normal mitochondria; red arrows marked swollen mitochondria. Scale bar: 1 μm (a–f). G, H Both A549 and SK-1 cells were pretreated with CCCP (10 or 5 μM) for 2 h, then treated with SFN (20 μM) for 24 h, the mitochondrial isolation kit was used to separate the cytoplasmic and mitochondrial proteins. The cytoplasm (G) or mitochondria (H) accumulation of LC3 II/I was analyzed by western blotting. I, J Both A549 and SK-1 cells were pretreated with Baf-A1 (50 nM) for 2 h, then treated with SFN (20 μM) for 24 h, the mitochondrial isolation kit was used to separate the cytoplasm and mitochondrial proteins. The cytoplasm (I) or mitochondria (J) accumulation of LC3 II/I was analyzed by western blotting. K, L After SFN was used at a designated concentration for 24 h, the mitochondrial isolation kit was used to separate the cytoplasmic and mitochondrial proteins. The binding of LC3 II/I to NIX was detected in both cytoplasm (K) and mitochondria (L) by coimmunoprecipitation. Data were shown as means ± SEM (n ≥ 3). *P ≤ 0.01; **P ≤ 0.001; ***P ≤ 0.0001.

Microtubule polymerization assay
The collected cells were washed twice with PBS, then lysed at 37 °C for 30 min with 400 μL lysis buffer (20 mM Tris-HCl pH 6.8, 1 mM MgCl2, 2 mM EGTA, 1% NP-40) with Protease Inhibitor Cocktail (Roche). The cell lysates were centrifuged at 12,500 r.p.m. for 15 min at 25 °C. The supernatant containing soluble α-tubulin was collected, whereas the pellet containing assembled α-tubulin was suspended in 40 μL of pellet lysis buffer (20 mM Tris-HCl pH 6.8, 1 mM MgCl2, 2 mM EGTA, 2% SDS) after washing with PBS. Then the precipitate was heated at 95 °C for 30 min until the pellet was solved. These α-tubulin molecules in two fractions (soluble and insoluble) were separated by western blotting.

Transmission electron microscopy
Cells were treated with 20 μM SFN for 24 h. After being collected and washed with PBS for two times, the sample was fixed with 3% glutaraldehyde at 4 °C for 2 h. After washing with PBS for three times, the sample was fixed in 1% osmium tetroxide for 1 h. Samples were infiltrated in Epon-812 for 2 h, cut into ultrathin sections with a knife, and positioned on 200-mesh copper grids. Sections were stained with Uranyl acetate for 30 min and then stained with Lead nitrate for 20 min. The sections were then observed and photographed with a TEM (JEM-2100Plus, JEOL, Ltd, Tokyo, Japan).

HPLC-MS/MS analysis
HPLC-MS/MS was used to analyze the production in A549 cells after treated with or without SFN. Cells were treated with SFN for 24 h, then the cell lysates were collected and quantified. Equal amount of protein molecules were analyzed by HPLC-MS/MS. Chromatographic separation and analysis were performed by a Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, USA) and an Agilent 1260 liquid chromatography system (Thermo Fisher Scientific, USA) equipped with an electrospray ionization source. Separation was carried out by a C18
(1.9 µm, 100 Å) capillary column with a mobile phase of 0.1% HCOOH/H₂O and 0.1% HCOOH/ACN. Each sample was injected twice to perform detection in positive ionization modes. After the collected data were identified, proteins were analyzed by the Uniprot net for cell localization and function.

**Tissue microarray immunohistochemistry**

Human lung cancer tissue microarrays with 150 patient samples and different Gleason patterns were established by Shanghai Biochip (Shanghai, China). The immunohistochemistry stain was done with human-specific anti-FASN combined with the UltraSensitive™ S-P detection
kit (Maixin, KIT-9710). The protocol was derived from the published paper [16].

**Apoptosis assay**

Cell apoptosis was measured via Annexin V-FITC apoptosis assay kit (GenStar, C203-01). Cells were collected and washed twice with ice-cold PBS, then these cells were resuspended in 250 μL 4× binding buffer. Then 5 μL Annexin V-FITC and 10 μL Propidium Iodide were added to 100 μL cell suspension, and the reaction was incubated for 5 min at room temperature in the dark. Apoptotic cells were detected via a flow cytometer.

**Free FA quantification assay**

Levels of total free FAs were determined by Free Fatty Acid Quantiﬁcation kit (Sigma, MAK044). Cells (1 × 10⁶) were collected and resuspended in 200 μL of 1% (w/v) Triton X-100 in chloroform solution. The homogenates were centrifuged at 13,000 r.p.m. for 10 min and we collected the organic phases to accept vacuum dry for 30 min, to remove trace chloroform. The dried lipids were dissolved in 200 L of Fatty Acid Assay Buffer for detection.

**Mitochondrial phospholipids quantification assay**

Cells (1 × 10⁶) were collected to isolate mitochondria with the kit. These mitochondria were lysed with 1% NP-40 for 30 min. Cell mitochondrial phospholipids were measured via Phospholipid Assay Kit (Sigma, MAK122).

**Bioinformatics analysis**

GEPIA (Gene Expression Profiling Interactive Analysis) is a public database newly developed by the Chinese for cancer and normal gene expression profiling, from 9736 tumors and 8587 normal samples from The Cancer Genome Atlas and Genotype-Tissue Expression projects. We searched the GEPIA Database to determine the genetic correlation in lung cancer. The version 10.5 of STRING database was used to find the interaction among NIX and lipid metabolism-related proteins [52].

**Statistical analysis**

All data were expressed as mean ± SEM from three independent experiments. Paired data were evaluated by Student’s t-test. Two-way analysis of variance was used to determine statistical signiﬁcance. P ≤ 0.05 was considered statistically signiﬁcant. All statistical analyses were performed by SPSS version 19.0.

**DATA AVAILABILITY**

All data generated or analyzed during this study are included in this published article.

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**AUTHOR CONTRIBUTIONS**

W.W. conceived, designed, and supervised the entire project. W.W., Y.T.Y., and Y.Z. developed the methodology. Y.T.Y., J.T.L., and Z.N.Z. acquired the data. Y.Z., Y.T., Y.B.H., and Z.N.Z. analyzed and interpreted the data (e.g., statistical analysis and computational analysis). W.W., Y.T.Y., and Y.Z. wrote and revised the manuscript. T.Z., Z.N.Z., J.T.L., and L.L. provided administrative and technical support (i.e., organizing data and constructing databases).

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**ETHICS STATEMENT**

This study is not involved in ethics.

**COMPETING INTERESTS**

The authors declare no competing interests.

**ADDITIONAL INFORMATION**

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