Role of Ser129 phosphorylation of α-synuclein in melanoma cells

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Summary
α-Synuclein, a protein central to Parkinson’s disease, is frequently expressed in melanoma tissues, but not in non-melanocytic cutaneous carcinoma and normal skin. Thus, α-synuclein is not only related to Parkinson’s disease, but also to melanoma. Recently, epidemiologists reported co-occurrence of melanoma and Parkinson’s disease in patients, suggesting that these diseases could share common pathogenetic components and that α-synuclein might be one of these. In Parkinson’s disease, phosphorylation of α-synuclein at Ser129 plays an important role in the pathobiology. However, its role in melanoma is not known. Here, we show the biological relevance of Ser129 phosphorylation in human melanoma cells. First, we have identified an antibody that reacts with Ser129-unphosphorylated α-synuclein but not with Ser129-phosphorylated α-synuclein. Using this and other antibodies to α-synuclein, we investigated the role of Ser129 phosphorylation in human melanoma SK-MEL28 and SK-MEL5 cells. Our immunofluorescence microscopy showed that the Ser129-phosphorylated form, but not the Ser129-unphosphorylated form, of α-synuclein localizes to dot-like structures at the cell surface and the extracellular space. Furthermore, immuno-electron microscopy showed that the melanoma cells release microvesicles in which Ser129-phosphorylated α-synuclein localizes to the vesicular membrane. Taken together, our studies suggest that the phosphorylation of Ser129 leads to the cell surface translocation of α-synuclein along the microtubule network and its subsequent vesicular release in melanoma cells.

Key words: Melanoma, Parkinson’s disease, α-Synuclein, Phosphorylation

Introduction
α-Synuclein (α-syn) is an important molecule clinically, since mutations and copy number variations in its gene have been linked to familial Parkinson’s disease (PD) (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004). In addition, inclusion bodies containing α-syn, referred to as Lewy bodies, are pathological hallmarks of familial and sporadic PD (Fujikawa et al., 2002; Tanji et al., 2006). Importantly, α-syn is predominantly phosphorylated at Ser129 (S129) in Lewy bodies of the PD brain (Fujikawa et al., 2002; Anderson et al., 2006). Thus, α-syn is a central molecule in understanding PD pathobiology, and its phosphorylation at S129 is thought to be involved in PD pathogenesis.

α-Syn is not only related to PD, but also to melanoma, which is the major cause of skin cancer death worldwide (Matsuo and Kamitani, 2010). We recently showed that α-syn is highly expressed in human melanoma cell lines (Matsuo and Kamitani, 2010; Lee and Kamitani, 2011). Moreover, α-syn is frequently expressed in human melanoma tissues, but it is undetectable in tissues of non-melanocytic cutaneous carcinoma and normal skin (Matsuo and Kamitani, 2010). These observations suggest that α-syn plays a role in the pathobiology of melanoma, as well as that of PD. Indeed, epidemiological studies have revealed co-occurrence of melanoma and PD in patients (Olsen et al., 2005; Olsen et al., 2006; Gao et al., 2009). Specifically, melanoma patients have an increased risk of subsequently developing PD (Olsen et al., 2006), and PD patients have an increased risk of subsequently developing melanoma (Olsen et al., 2005). Therefore, it is possible that α-syn is shared as a common pathogenetic component in these two diseases (Matsuo and Kamitani, 2010).

α-Syn is a small soluble protein containing 140 amino acids (17 kDa), and predominantly expresses in the brain. α-Syn localizes to synaptic vesicles on the presynaptic side and the nucleus – hence the name ‘synuclein’ (Maroteaux et al., 1988; Auluck et al., 2010). Although the exact function of α-syn is still unclear, substantial evidence now exists to suggest that this protein is predominantly natively unfolded in solution but can bind to phospholipid membranes by adopting an amphipathic helical conformation in its N-terminal amino acids (Bennett, 2005; McFarland et al., 2008).

α-Syn has at least three experimentally proven phosphorylation sites, S87, S129 and Y125 (Chau et al., 2009). Among these sites, S129 has been studied most in the neuropathology field as described above. With regard to the physiological role of the S129 phosphorylation, there have been several reports. Pronin et al. reported that the S129 phosphorylation leads to the dissociation of α-syn from lipids (Pronin et al., 2000). Lou et al. reported that the S129 phosphorylation reduces the ability of α-syn to regulate tyrosine hydroxylase (TH) and protein phosphatase 2A (Lou et al., 2009).
Recently, McFarland et al. showed that the C-terminal peptide of S129-phosphorylated α-syn interacts preferentially with cytoskeletal proteins and vesicular trafficking proteins (McFarland et al., 2008), suggesting that the S129 phosphorylation likely has a profound effect on protein trafficking. Despite these observations, the physiological relevance of S129 phosphorylation is still unclear.

In this report, we investigated a novel, physiological role of the S129 phosphorylation in human melanoma SK-MEL28 and SK-MEL5 cells. We first identified an antibody specific to S129-unphosphorylated α-syn. Using this as well as an antibody specific to S129-phosphorylated α-syn, we investigated the subcellular localization of α-syn and found that the S129-phosphorylated form localizes to dot-like structures at the cell surface and the extracellular space. On the basis of these results and others, we have proposed that the phosphorylation of S129 plays a role in the translocation to the cell surface and subsequent vesicular release of α-syn in melanoma cells.

Results

4D6 reacts with amino acid residues 124–134 of human α-syn

4D6 is a mouse monoclonal anti-α-syn antibody (Golovko et al., 2005), but its epitope had not been identified. We therefore performed epitope mapping. We expressed several deletion mutants of α-syn as fusion proteins in yeast cells and tested for reactivity with 4D6 using western blotting (Fig. 1A). The results mapped the epitope to within 11 amino acid residues (124–134) at the C-terminal region containing the S129 phosphorylation site (see lane 9). The results are summarized in Fig. 1B,C.

4D6 selectively recognizes the S129-unphosphorylated form of α-syn

We next determined whether phosphorylation at S129 affects the 4D6 immunoreactivity to α-syn. We performed western blot analysis using both unphosphorylated and phosphorylated forms of α-syn. In brief, α-syn was expressed in bacterial cells and purified. The unphosphorylated recombinant α-syn protein was treated with casein kinase-2 (CK2), which is a serine/threonine-purified. The unphosphorylated recombinant α-syn protein was treated with casein kinase-2, which is a serine/threonine protein kinase (Fujiwara et al., 2002), to generate treated with casein kinase-2 (CK2), which is a serine/threonine- a

4D6 selectivity was determined by western blotting using anti-α-syn monoclonal antibodies LB509 [for total α-syn (Jakes et al., 1999)], EP1536Y [for S129-phosphorylated α-syn (Fournier et al., 2009; Qing et al., 2009)], and 4D6. As shown in Fig. 2A, LB509 detected both unphosphorylated and phosphorylated forms of α-syn. EP1536Y only detected α-syn that was phosphorylated by CK2. 4D6 detected α-syn that was not phosphorylated by CK2, but did not detect α-syn that was phosphorylated by CK2, suggesting that 4D6 selectively reacts with the S129-unphosphorylated form of α-syn.

We further confirmed the immunoreactivity of 4D6 to S129-unphosphorylated α-syn using another method. Specifically, α-syn (without tag) was overexpressed in SH-SY5Y cells (Chau et al., 2009) and resolved by two-dimensional gel electrophoresis, followed by western blot analysis using anti-α-syn antibodies LB509 (for total α-syn), EP1536Y (for S129-phosphorylated α-syn), and 4D6. As shown in Fig. 2B, LB509 detected at least five species of α-syn (~17 kDa) at the pH range between 4.4 and 5.0. Since EP1536Y only detected spot no. 1, we identified spot no. 1 as S129-phosphorylated α-syn and spots no. 2–5 as S129-unphosphorylated α-syn.

Role of S129 phosphorylation in α-syn localization in human melanoma SK-MEL28 cells

α-Syn is predominantly expressed in neurons and localizes to synaptic vesicles and portions of the nucleus (Maroteaux et al., 1988). Recently, we found that α-syn is expressed more in human melanoma tissues and cell lines (Matsuo and Kamitani, 2010; Lee and Kamitani, 2011). However, its subcellular localization in melanoma cells has not been known. To determine this, we immunostained human melanoma SK-MEL28 cells using 4D6 antibody for α-syn unphosphorylated at S129 (Fig. 3A) and pSyn

![Image](https://via.placeholder.com/150)

Fig. 1. Epitope mapping of 4D6 antibody on α-syn. (A) 4D6 immunoreactivity with deletion mutants of α-syn. To express AD-HA epitope alone and AD-HA-fused α-syn (full-length and deletion mutants), yeast cells were transformed and grown in a selection medium. Their total cell lysates were then prepared and analyzed by western blotting. Anti-HA antibody 16B12 (top panel) and anti-α-syn antibody 4D6 (middle panel) were used to determine the immunoreactivity with AD-HA (lanes 1 and 6) and AD-HA-fused α-syn (lanes 2–5 and 7–8). To demonstrate equal loading amounts of total cell lysates, western blotting using anti-actin antibody was also performed (bottom panel). The length of wild-type and deletion mutants of α-syn are indicated by numbers of amino acid residues (e.g. 1–140 for the wild-type α-syn, 1–50 for the C-terminal deletion mutant and 90–140 for the N-terminal deletion mutant). Molecular size markers are shown in kilodaltons. (B) Summary of 4D6 immunoreactivity on full-length α-syn and its deletion mutants. Amino acid residues 1–60, amphipathic region; amino acid residues 61–95, NAC (non-Aβ component of Alzheimer’s disease amyloid) region; and amino acid residues 96–140, acidic region. S129 residue for phosphorylation is indicated by an arrowhead. (C) Recognition sites of 4D6 and other anti-α-syn antibodies (EP1536Y, LB509 and pSyn#64) on α-syn. Recognition sites of antibodies are indicated by arrows.
Fig. 2. 4D6 immunoreactivity with unphosphorylated and phosphorylated forms of α-syn. (A) 4D6 immunoreactivity with kinase-ununtreated and treated α-syn. Recombinant human α-syn was purified from bacterial culture and incubated with or without protein kinase CK2. Unphosphorylated and phosphorylated forms of α-syn were then analyzed by western blotting using anti-α-syn antibody LB509 (top panel), anti-phospho-α-syn antibody EP1536Y (middle panel) and anti-α-syn antibody 4D6 (bottom panel). Molecular size markers are shown in kilodaltons. (B) Specific immunoreactivity of 4D6 with S129-unphosphorylated form of α-syn expressed in SH-SY5Y cells. α-Syn (without tag) was overexpressed in SH-SY5Y cells by plasmid transfection. The total cell lysate was resolved by two-dimensional gel electrophoresis, and western-transferred to a membrane. Using a stripping method, the membrane was then serially probed with mouse monoclonal anti-α-syn antibody LB509 (top panel), rabbit monoclonal anti-phospho-α-syn antibody EP1536Y (middle panel) and mouse monoclonal anti-α-syn antibody 4D6 (bottom panel) for western blot analysis. Molecular size markers are shown in kilodaltons.

Fig. 3. Subcellular localization of S129-unphosphorylated and phosphorylated forms of endogenous α-syn in human melanoma SK-MEL28 cells. (A) Double immunostaining of SK-MEL28 cells with anti-α-syn antibody 4D6 (for S129-unphosphorylated form) and anti-NUB1 antibody (for counterstaining). (B) Double immunostaining of SK-MEL28 cells with anti-phospho α-syn antibody pSyn#64 (for S129-phosphorylated form) and anti-NUB1 antibody (for counterstaining). (C) Comparison between 4D6 immunostaining and pSyn#64 immunostaining. Human melanoma SK-MEL28 cells were double-immunostained with mouse monoclonal anti-α-syn antibody (4D6 or pSyn#64) and rabbit polyclonal anti-NUB1 antibody. Cells were then labeled with fluorescent dye-conjugated secondary antibodies. The stained cells were analyzed by fluorescence microscopy. In A and B, the localization of endogenous α-syn is shown by the green fluorescence (left panels). The localization of endogenous NUB1 is shown by the red fluorescence. Both images are merged in the middle panels. The framed areas (middle panels) are magnified in the right panels. Scale bars indicate 20 μm in the left and middle panels and 5 μm in the right panels. In C, only merged images are shown with nuclear DAPI staining (the blue fluorescence). Scale bars indicate 20 μm.

demonstration in the immunofluorescence microscopy. However, the dot-like structure with S129-phosphorylated α-syn clearly existed around the cells (which looked like a starry sky), suggesting that the phosphorylation of α-syn at S129 is involved in its cell surface localization and subsequent release.

Immunoreactivity of 4D6 and pSyn#64 antibodies to α-syn-knockdown SK-MEL28 cells

As described above, we observed differences in 4D6 and pSyn#64 immunostainings. To confirm that the differences do
not result from non-specific reaction of antibodies, we examined the immunoreactivity of 4D6 and pSyn#64 to α-syn-knockdown SK-MEL28 cells. For this purpose, we stably knocked down endogenous α-syn in SK-MEL28 cells by using lentivirus-mediated RNAi. In brief, SK-MEL28 cells were infected with lentiviruses to express five different sequence-verified shRNAs. We then chose one SK-MEL28 cell line (out of five cell lines) whose average diameter is 150 nm (arrows, Fig. 7B–D). As shown in the upper panel of Fig. 5A, the expression of endogenous α-syn was interfered in all cell lines expressing α-syn shRNA (no. 1–5). Because endogenous α-syn was strongly knocked down in cell line no. 3, we chose this cell line for further experiments. In addition to the cell line no. 3, we established a cell line expressing control shRNA (first lane).

Next, we immunostained these SK-MEL28 cell lines with 4D6 and pSyn#64 antibodies. As shown in Fig. 5B, cells expressing control shRNA were strongly immunostained with both α-syn antibodies 4D6 (upper panels) and pSyn#64 (lower panels). The localization of α-syn is shown by the green fluorescence. Nuclei are counterstained blue by DAPI. Scale bars indicate 50 μm.

Fig. 5. Immunoreactivity of 4D6 and pSyn#64 antibodies to α-syn-knockdown SK-MEL28 cells. (A) Expression levels of α-syn in SK-MEL28 cells expressing shRNA of α-syn. Total cell lysates were prepared from cells expressing control shRNA (first lane) and cells expressing α-syn shRNA (no. 1–no. 5). These lysates were analyzed by western blotting using anti-α-syn antibody 4D6 (upper panel) and anti-actin antibody (lower panel). Molecular size markers are shown in kilodaltons. (B) Immunostaining of α-syn-knockdown SK-MEL28 cells. Cells expressing control shRNA (left panels) and those expressing α-syn shRNA (right panels) were immunostained with anti-α-syn antibodies 4D6 (upper panels) and pSyn#64 (lower panels). The localization of α-syn is shown by the green fluorescence. Nuclei are counterstained blue by DAPI. Scale bars indicate 20 μm.

Ultrastructural localization of endogenous α-syn phosphorylated at S129 in human melanoma SK-MEL28 cells

Next, we performed immuno-electron microscopy to investigate the ultrastructural localization of S129-phosphorylated α-syn at the cell surface and underneath the plasma membrane. As shown in Fig. 7, immuno-electron microscopy revealed that S129-phosphorylated α-syn localizes to small structures (40 to 60 nm in diameter) underneath the plasma membrane (arrows, Fig. 7A). We speculate that S129-phosphorylated α-syn is either oligomerized into small aggregates or accumulated within the 40–60 nm structures. In addition, S129-phosphorylated α-syn localizes to the membranes of MVs, whose average diameter is ~150 nm (arrows, Fig. 7B–D). Thus, our immuno-electron microscopy revealed that SK-MEL28 cells release S129-phosphorylated α-syn as MVs.

Role of S129 phosphorylation in α-syn localization in various melanoma cell lines

In human melanoma SK-MEL28 cells, S129-phosphorylated α-syn localized to the cell surface as well as the nucleus. To
determine whether this is observed in other human melanoma cell lines, we tested the SK-MEL5, A375, MeWo and WM266-4 melanoma cell lines. First, we examined the expression levels of endogenous α-syn using different antibodies, including LB509 (for total α-syn), 4D6 (for S129-unphosphorylated α-syn), pSyn#64 and EP1536Y (for S129-phosphorylated α-syn). As shown in Fig. 8A, SK-MEL5, MeWo and WM266-4 cells, as well as SK-MEL28 cells, expressed both S129-unphosphorylated and phosphorylated forms. Notably, SK-MEL5 cells expressed them at much higher levels. However, the expression levels were extremely low in A375 cells as we described previously (Lee and Kamitani, 2011).

Next, we immunostained HT1080 (as negative control), SK-MEL5, MeWo and WM266-4 cells using anti-α-syn antibodies 4D6 and pSyn#64. A375 cells were not used because S129-phosphorylated α-syn was almost undetectable by western blotting. As shown in Fig. 8B, S129-unphosphorylated α-syn localized to the cytoplasm as small dots, but its nuclear localization was extremely limited in three melanoma cell lines (top panels). In contrast, S129-phosphorylated α-syn localized to the nucleus and cytoplasm in these cell lines (middle and bottom panels). It should be noted that it also localized to the cell surface in SK-MEL5 cells, but not in MeWo and WM266-4 cells (see the magnified images at the bottom). In supplementary material Fig. S1, we showed additional SK-MEL5 cells immunostained with 4D6 or pSyn#64 to support these findings. Thus, we found that S129-phosphorylation plays a role in localization of α-syn to the cell surface in two (SK-MEL5 and SK-MEL28) out of five human melanoma cell lines tested in this study.

Moreover, we showed that the dot-like structures with S129-phosphorylated α-syn existed around SK-MEL5 cells (data not shown), suggesting that the phosphorylation of α-syn at S129 is involved in its cell surface localization and subsequent release in SK-MEL5 as well as SK-MEL28 cells (also see Fig. 4).

Translocation of α-syn-positive structures along the microtubule network

Previously, tubulin was revealed to be an α-syn-binding protein (Alim et al., 2004). In neurons, α-syn was shown to be transported by both kinesin and dynein motor proteins along microtubules (Utton et al., 2005). These observations suggested that a transport system along microtubules is involved in translocation of α-syn in melanoma cells. To test this possibility, we compared the distributions of dot-like structures of α-syn and the microtubule network in melanoma cells. Specifically, we double-immunostained endogenous α-syn (unphosphorylated or phosphorylated at S129) and endogenous α-tubulin of the microtubule network in flat extended SK-MEL5 cells to determine their distributions by fluorescence microscopy. As shown in the upper panels of Fig. 9, α-tubulin formed a fine network structure of microtubules, while S129-unphosphorylated α-syn was observed as dot-like structures. When the images were merged, the microtubule network was abundantly decorated with dot-like structures of α-syn (Fig. 9A). Importantly, the magnified image clearly showed that the dot-like structures are located along the microtubules (Fig. 9B). In contrast, S129-phosphorylated α-syn localized to the nucleus and cell surface...
Fig. 8. Localization of S129-unphosphorylated and phosphorylated forms of endogenous α-syn in various human melanoma cell lines. (A) Expression levels of S129-unphosphorylated and phosphorylated α-syn. Total cell lysates were prepared from human melanoma cell lines, SK-MEL28, SK-MEL5, A375, MeWo and WM266-4, and human lung fibrosarcoma cell line HT1080 (negative control). The lysates were analyzed by western blotting using anti-α-syn antibodies LB509 (for total α-syn), 4D6 (for S129-unphosphorylated form), pSyn#64 (for S129-phosphorylated form) and EP1536Y (for S129-phosphorylated form). In addition, expression levels of NUB1 and actin were examined. Molecular size markers are shown in kilodaltons. (B) Double immunostaining of HT1080 and three melanoma cell lines with anti-α-syn antibody (4D6 or pSyn#64) and anti-NUB1 antibody (for counterstaining). The localization of endogenous α-syn is shown by the green fluorescence. The localization of endogenous NUB1 is shown by the red fluorescence. Nuclear counterstaining is shown by the blue fluorescence of DAPI. These three-color images are merged in all panels. The framed areas (middle panels) are magnified in the bottom panels. Scale bars indicate 20 μm in the top and middle panels and 5 μm in the bottom panels.

Fig. 9. Double immunostaining of microtubules and α-syn in human melanoma SK-MEL5 cells. Cells were fixed in cold methanol, permeabilized with 0.1% Triton X-100, and immunostained with both rat monoclonal anti-α-tubulin antibody and mouse monoclonal anti-α-syn antibody (4D6 or pSyn#64). After washing, the cells were labeled with both Alexa Fluor 488-conjugated anti-rat IgG antibody and Alexa Fluor 555-conjugated anti-mouse IgG antibody. The cells were then stained with DAPI and analyzed by fluorescence microscopy. The localization of microtubules is shown by the green fluorescence of Alexa Fluor 488, and the localization of endogenous α-syn is shown by the red fluorescence of Alexa Fluor 555. Nuclear counterstaining is shown by the blue fluorescence of DAPI. (A,B) double immunostaining with 4D6 and anti-α-tubulin antibody. (C,D) double immunostaining with pSyn#64 and anti-α-tubulin antibody. Panels A and C are magnified in panels B and D, respectively. Scale bars indicate 20 nm (A,C) and 5 nm (B,D).

Discussion

α-Syn has at least three experimentally proven phosphorylation sites (Chau et al., 2009), of which the S129 residue is thought to be the major site (Wang et al., 2012). The phosphorylation of S129 plays an important role in the pathobiology of neurodegenerative α-synucleinopathy, because it is selectively and extensively phosphorylated in α-synucleinopathy lesions such as the Lewy bodies and Lewy neurites found in the PD brain (Fujisawa et al., 2002; Anderson et al., 2006). However, little is currently known of the physiological (not pathological) relevance of this phosphorylation, partly due to the technological difficulties of the required experiments. Specifically, to clearly determine the physiological role of S129 phosphorylation, we have to examine S129-unphosphorylated α-syn as well as S129-phosphorylated α-syn. For this purpose, two different antibodies to α-syn are needed: one antibody for α-syn unphosphorylated at S129 and another antibody for α-syn phosphorylated at S129. However, although the latter antibody is commercially available, no antibody for S129-unphosphorylated α-syn had previously been identified, making it difficult to study the physiological role of S129 phosphorylation.

In our present study, we identified 4D6 as an antibody to S129-unphosphorylated α-syn. Importantly, 4D6 recognizes the same C-terminal region as pSyn#64 does. Unlike 4D6, however, pSyn#64 is an antibody to α-syn phosphorylated at S129 and has commonly been used for immunostaining to determine the localization of S129-phosphorylated α-syn (Fujisawa et al., 2002; Tanji et al., 2006; Tanji et al., 2011). Therefore, we expected that immunostaining using both 4D6 and pSyn#64 antibodies would allow us to more clearly and exactly uncover the role of S129 phosphorylation in the subcellular localization of α-syn. Using these antibodies, we immunostained human melanoma SK-MEL28 and SK-MEL5 cells that highly express endogenous α-syn (Matsuo and Kamitani, 2010; Hansen et al., 2011; Lee and
The immunostaining showed that S129-phosphorylated α-syn localizes to the cell surface as small dots, but S129-unphosphorylated α-syn does not. Importantly, we also detected the released dot-like structures containing S129-phosphorylated α-syn by using immunofluorescence microscopy. Furthermore, immuno-electron microscopy revealed that SK-MEL28 melanoma cells release MVs in which S129-phosphorylated α-syn localizes to the vesicular membrane. These results suggest that the S129 phosphorylation plays a role in the cell surface localization and subsequent vesicular release of α-syn in some of the human melanoma cells.

α-Syn release has intensively been studied using neuronal, but not melanoma, cells. These studies revealed that α-syn is released from neuronal cells in a constitutive manner (Lee et al., 2005; Lee et al., 2008). This is supported by a previous clinical finding that α-syn is present in human cerebrospinal fluid at low nanomolar concentrations (Borghi et al., 2000; El-Agnaf et al., 2003). Since both neuronal cells and melanocytes developmentally originate from the neural crest and share several features, the α-syn release may be a common feature in both cell types.

How is α-syn release regulated in melanoma cells? As described above, our immunofluorescence microscopy revealed that the dot-like structures are immunostained by pSyn/64 antibody, but not by 4D6 antibody, at the cell surface and outside of melanoma cells. These results suggest that S129 phosphorylation regulates the α-syn release in melanoma cells. Indeed, McFarland et al. demonstrated that S129 phosphorylation enables α-syn to interact with vesicular trafficking proteins of mouse brain (McFarland et al., 2008). Most recently, Wang et al. detected the selective elevation of S129-phosphorylated α-syn in the cerebrospinal fluid of PD patients (Wang et al., 2012). On the basis of these observations and our results, S129 phosphorylation might regulate the release of α-syn through the vesicular trafficking proteins in melanoma cells as well as neuronal cells. Importantly, our double immunostaining of α-syn and β-tubulin suggested that, upon phosphorylation at S129, α-syn is trafficked along the microtubule network.

What is the biological and clinical relevance of the α-syn release in melanoma? Although not known for melanoma, this has been well investigated in neuronal cells, and an interesting hypothesis has been proposed for α-syn release in the pathogenesis of PD. In this hypothesis, molecules of α-syn with an abnormal structure are released from one neuron and taken up by another, leading to neurodegeneration by prion-like mechanisms (Frost and Diamond, 2010; Olanow and McNaught, 2011; Steiner et al., 2011). In melanoma, epidemiological studies have revealed co-occurrence of melanoma with PD (Olsen et al., 2005; Olsen et al., 2006; Gao et al., 2009). Specifically, melanoma patients have an increased risk of subsequently developing PD (Olsen et al., 2006). Therefore, it is possible that α-syn release from melanoma cells plays a role in the pathogenesis or progression of PD, if it is propagated into neuronal cells. Indeed, this possibility is partially supported by a recent observation that α-syn is propagated from human melanoma SK-MEL5 cells to mouse neuroblastoma N2a cells in co-culture conditions (Hansen et al., 2011).

In conclusion, we have reported that the S129-phosphorylated form, but not the S129-unphosphorylated form, of α-syn localizes to dot-like structures at the cell surface and the extracellular space of melanoma cells. Our studies suggest that the phosphorylation of S129 leads to the cell surface translocation of α-syn along the microtubule network and subsequent vesicular release in melanoma cells. Further studies will be required to determine the clinical relevance of this finding.

**Materials and Methods**

**Cell lines and culture conditions**

The following human cell lines were purchased from American Type Culture Collection (Manassas, VA): SH-SYSY (human neuroblastoma), SK-MEL28 (human malignant melanoma), SK-MEL5 (human malignant melanoma), A375 (human malignant melanoma), WM266-4 (human malignant melanoma), and HT1080 (human lung fibrosarcoma). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics.

**Antibodies**

Rat monoclonal anti-β-tubulin (YLI/12) antibody and mouse monoclonal anti-α-syn antibodies LB509 (specific for amino acid residues 115–122) and 4D6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-phospho α-syn antibody pSyn/64 (specific for amino acid residues 124–134) was purchased from Wako Chemicals USA (Richmond, VA). Rabbit monoclonal anti-phospho α-syn antibody EP1536Y (specific for amino acid residues 120–140) was purchased from Epitomics ( Burlingame, CA). Mouse monoclonal anti-HA antibody 16B12 was purchased from Covance (Richardson, CA). Rabbit polyclonal anti-NUB1 antibody was generated by immunization with a GST fusion protein of NUB1, corresponding to amino acid residues 432–601 (Kito et al., 2001).

**Yeast transformation and cell lysate preparation**

For epitope mapping of mouse monoclonal anti-α-syn antibody 4D6, several truncated fragments of α-syn were expressed in yeast cells and detected by western blotting using 4D6. In brief, the cDNAs of truncated α-syn were amplified by polymerase chain reaction (PCR) from pcDNA3/α-syn (see Two-dimensional gel electrophoresis below) using appropriate primers. These cDNAs were inserted into the multi-cloning site of yeast expression vector pGADT7 (Clontech, Mountain View, CA). Since the multi-cloning site was located downstream of the cDNA encoding Gal4-activating domain (AD) and HA tag, the plasmids allow us to express AD-HA-fused α-syn fragments in yeast cells. For the expression, AH109 yeast cells were transformed with the plasmids using the lithium acetate method (Okura et al., 1996). Transformed cells were grown on a Leu- synthetic agar plate for 3 days at 30˚C. The yeast cells were then grown in a Leu- synthetic medium. Afterwards, using the boiling-SDS-glass bead method (Matsuo et al., 2004), the total cell lysate was prepared for western blotting.

**Purification of recombinant human α-syn from bacterial culture**

The cDNA of wild-type α-syn was subcloned into pTI7-7 expression plasmid vector (USB, Cleveland, OH) for inducible expression in Escherichia coli. Rosetta (DE3) expression host cells (Novagen, Madison, WI) were then transformed with the plasmid and grown in 1 l of LB medium containing ampicillin and chloramphenicol at 37˚C with shaking. When the optical density of the culture medium reached 0.6 at 600 nm, α-syn was induced by adding 1 mM isopropyl thiogalactoside, followed by an additional culture for 3 h. Cells were harvested by centrifugation and the pellet was subjected to purification essentially as described previously (Volles and Lansbury, 2007). Briefly, cells were lysed by boiling in the buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl]. Nucleic acids were removed by precipitation with streptomycin sulfate and acetic acid. α-Syn was subsequently purified by ammonium sulfate precipitation. The pellet was resuspended in ammonium acetate, followed by ethanol precipitation three times. The final pellet was resuspended in ammonium acetate, aliquoted into appropriate amounts, subjected to lyophilization, and stored until use for “in vitro phosphorylation assay”.

**In vitro phosphorylation of α-syn**

Recombinant human α-syn (3.5 μg) was incubated in 10 μl of in vitro phosphorylation buffer [20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl2, 5 mM ATP] (Fujiwara et al., 2002) with or without 500 units of purified casein kinase-2 (CK2; New England Biolabs, Ipswich, MA) for 18 h at 30˚C. After the kinase reaction, protein samples were heated for 1 h in 2% SDS treating solution and analyzed by western blotting.

**Western blot analysis**

Protein samples were heated for 30 min in a sample-treating solution containing 2% SDS and 5% β-mercaptoethanol. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, Immobilon P (Millipore, Bedford, MA). Western blotting was then performed as described previously (Matsuo and Kamitani, 2010; Lee and Kamitani, 2011). Horseradish peroxidase (HRP)-conjugated antibodies against mouse IgG or rabbit IgG (Santa Cruz Biotechnology) were used as secondary antibodies.
Release of α-synuclein from melanoma

0.1 M NaCac buffer (pH 7.4) containing 2% osmium tetroxide for 1 h, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series, and embedded in epon-araldite resin. Thin sections were cut with a diamond knife on an EM UC6 ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL), collected on copper grids, and sequentially stained with 2% uranyl acetate for 5 min and Reynolds lead citrate for 3 min. The sections were observed by a JEM 2100 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV and imaged with UltraScan 4000 CCD Camera and First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA).

**Immunoelectron microscopy**

SK-MEL28 cells were fixed on a culture dish for 3 min in 0.1 M NaCac buffer (pH 7.4) containing 4% paraformaldehyde and 0.2% glutaraldehyde, scraped with a cell scraper, and pellets by centrifugation. After 1 h, the cells were dehydrated with a graded ethanol series and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut with a diamond knife on an EM UC6 ultramicrotome and collected on nickel grids. Sections were incubated in the blocking buffer [50 mM Tris- HCl (pH 7.4), 118 mM NaCl, 10 mM NaF, 5% BSA, 3% normal donkey serum, 0.05% Tween-20] at room temperature for 2 h, followed by incubation with mouse monoclonal anti-phospho α-syn antibody pSyn#64 (dilution 1:75) overnight at 4°C. The sections were washed and stained for 2 h at room temperature with donkey anti-mouse IgG (Abcam, Cambridge, MA) at 1:1000 and then incubated with colloidal gold particles (15 nm in diameter) as described previously (Slot and Geuze, 1985). After washing, the sections were stained with 2% uranyl acetate alone for the cytoplasm (Fig. 7A) or with 2% uranyl acetate and 0.04% bismuth suburate sequentially for cellular surface structures (Ainsworth et al., 1972) (Fig. 7B-D). The stained sections were then observed by a JEM 1230 transmission electron microscope as described above.

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**References**


**Electron microscopy**

SK-MEL28 cells were fixed with 0.1 M sodium cacodylate (NaCac) buffer (pH 7.4) containing 2% glutaraldehyde for 3 min on a culture dish, scraped with a cell scraper, and pellets by centrifugation. After 1 h, the cells were postfixed in 2% glutaraldehyde for 3 min on a culture dish, scraped with a cell scraper, and pellets by centrifugation.


Fig. S1. Subcellular localization of S129-unphosphorylated and phosphorylated forms of endogenous α-syn in human melanoma SK-MEL5 cells. (A) Double immunostaining of SK-MEL5 cells with anti-α-syn antibody 4D6 (for S129-unphosphorylated form) and anti-NUB1 antibody (for counterstaining). (B) Double immunostaining of SK-MEL5 cells with anti-phospho α-syn antibody pSyn#64 (for S129-phosphorylated form) and anti-NUB1 antibody (for counterstaining). Human melanoma SK-MEL5 cells were double-immunostained with mouse monoclonal anti-α-syn antibody (4D6 or pSyn#64) and rabbit polyclonal anti-NUB1 antibody. Cells were then labeled with fluorescent dye-conjugated secondary antibodies. The stained cells were analyzed by fluorescence microscopy. The localization of endogenous α-syn is shown by the green fluorescence. The localization of endogenous NUB1 is shown by the red fluorescence. Nuclear counterstaining is shown by the blue fluorescence of DAPI. These images are merged in all panels. The framed areas (upper panels) are magnified in the lower panels. Scale bars indicate 20 µm in the upper panels and 5 µm in the lower panels.