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Title

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Permalink

https://escholarship.org/uc/item/6sd5b5pm

Journal

European Journal of Human Genetics, 23(8)

ISSN

1018-4813

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Publication Date

2015-08-01

DOI

10.1038/ejhg.2015.45

Peer reviewed

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Clinical and ultrastructural spectrum of diffuse lung disease associated with surfactant protein C mutations

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Running title:
Histopathology and ultrastructure in SP-C mutants

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Abstract

Genetic defects of surfactant metabolism are associated with a broad range of clinical manifestations, from neonatal respiratory distress syndrome to adult interstitial lung disease. Early therapies may improve symptoms but diagnosis is often delayed due to phenotype and genotype variability. Our objective was to characterize the cellular/ultrastructural correlates of surfactant protein-C mutations in children with idiopathic diffuse lung diseases. We sequenced *SFTPC* -the gene encoding surfactant protein C-, *SFTPB* and *ABCA3*, and analyzed morphology, ultrastructure and surfactant protein expression in lung tissue when available. We identified eight subjects who were heterozygous for SP-C mutations. Median age at onset and clinical course was variable. None of the mutations were located in the mature peptide-encoding region, but were either in the pro-protein BRICHOS or linker C-terminal domains. While lung morphology was similar to other genetic surfactant metabolism disorders, electron microscopy studies showed specific anomalies suggesting surfactant homeostasis disruption, plus trafficking defects in the four subjects with linker domain mutation and protein misfolding in the single BRICHOS mutation carrier in whom material was available. Immunolabeling studies showed increased proSP-C staining in all cases. In two cases, amyloid deposits could be identified. Immunochemistry and ultrastructural studies may be useful for diagnostic purposes and for genotype interpretation.

Keywords: Surfactant protein C, diffuse parenchymal lung disease, mutation, lamellar bodies, amyloid, BRICHOS domain.

Background

Pediatric diffuse parenchymal lung diseases (pDLD) are rare disorders with various etiologies, characterized by chronic or progressive gas exchange impairment and multifocal or diffuse infiltrates. Mutations in several surfactant-related genes have been increasingly recognized as causes of pDLD [1]; these genes encode different types of protein: intrinsic surfactant peptides (surfactant protein B [SP-B] and C [SP-C] [2]), transmembrane transporters (ATP-binding cassette protein A3 [ABCA3] [3]), gene expression regulators - thyroid transcription factor 1 [TTF-1]), receptors -granulocyte macrophage colony stimulating factor receptor subunits a and b [CSF2Rα and CSF2Rβ]), accounting in part for the extreme diversity of the clinical spectrum. SP-C is a small hydrophobic peptide, which, in association with SP-B, plays a key role in surface film formation and stability at the alveolar gas-liquid interface [4]. The *SP-C* gene, also designated *SFTPC*, is located at the 8p21 locus, spanning 3.5 kb, is composed of 6 exons, and encodes a 191 or 197 aminoacid (aa) apoprotein depending upon alternative splicing, that subsequently traffics through the endoplasmic reticulum and the Golgi and undergoes cleavage of N- and C-terminus residues in multivesicular bodies (MVBs), leading to formation of the 35 aa mature SP-C peptide [5]. This peptide interacts with SP-B and phospholipids inside the lamellar bodies (LBs) to form bioactive surfactant. The N-terminus domain is involved in cell trafficking and the C-terminus domain harbors the BRICHOS domain, a chaperone preventing aggregation of the hydrophobic mature peptide during post-transcriptional processes. SP-B and ABCA3 deficiencies are autosomal recessive diseases presenting as neonatal respiratory distress syndrome (RDS) typically fatal within the first months of age (although certain ABCA3 mutations may lead to later onset disease [6]). SP-C mutations are mostly mono-allelic, either sporadic or inherited and are expressed in a dominant fashion in about 50% of cases.

Clinical onset varies from birth to advanced adulthood, with variable severity and outcome [7]. Complex molecular mechanisms account in part for the phenotypic diversity of the disease. Environmental factors also play a key role in modulating the disease course, as carriers of the same mutation in a single pedigree may show a broad variability of onset and presentation [8,9]. Lung infections often trigger or complicate the course of pDLD in SP-C mutation carriers, especially in infantile age. In the context of an ongoing research project aimed at determining the contribution of rare and common variants of surfactant-related genes in pDLD, we report the clinical features and molecular and histological findings in eight subjects heterozygous for *SFTPC* rare variants.

Methods

Subjects. From 2005 to 2012, infants and children with a history of unexplained persistent or progressive diffuse lung disease based on clinical signs (abnormal auscultation, cough, dyspnea, hypoxemia) with an onset between birth and 18 years plus evidence of diffuse parenchymal lung disease were referred to our center for genetic testing. Exclusion criteria consisted of acute/chronic airway infections, cystic fibrosis, aspiration pneumonia, immune deficiencies, primary ciliary dyskinesia, tuberculosis and allergic bronchopulmonary aspergillosis. Parental consent was obtained when applicable. We sequenced *SFTPC, SFTPB* and *ABCA3,* plus other genes in selected cases based on family history and clinical findings (online supplement). Patient #7 was referred to our center as a young adult, but was included in this series as she had respiratory symptoms since one year of age. The study was conducted in compliance with the hospital internal review board and research ethics committee.

DNA analysis. We performed PCR-based mutation analysis of the *SFTPC*, *SFTPB* and *ABCA3* genes by Sanger's technique. All coding exons were amplified by PCR with primers synthesized for the coding and flanking regions of each gene using the human genome sequences ENSG00000168484 *(SFTPC)*, ENSG00000168878 *(SFTPB)* and ENSG00000167972 *(ABCA3)*, and were sequenced bidirectionally [10]. Amino acid sequences were expressed following the NCBI NP_001165881 (proSP-C) and NP_001080.2 (ABCA3) reference sequences. Familial DNA was sequenced when available, and rare variants were tested in 100 alleles and in the 1000 Genomes Project phase1 NHBLI Exome sequencing project (1000G) and NHLBI Grand Opportunity Exome Sequencing Project (ESP) databases. The impact of predicted amino-acid changes on protein structure and function was assessed by SIFT [11] and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2). Aa conservation was assessed by sequence comparison in 28 vertebrate species. The *SFTPC* variants were submitted to the Leiden Open Variation Database (LOVD) public database (http://www.lovd.nl); identification numbers are provided in table 2. For previously described variants, we also provided the identification number in the dbSNP public database when available.

Optical microscopy. Sections of formaldehyde-fixed paraffin-embedded lung tissue were labeled with 1:100 mouse anti SP-B antibody (Lab Vision, Fremont, CA) or 1:400 polyclonal rabbit anti proSP-C (Millipore, Temecula, CA) [12] and detected with peroxidase-coupled secondary antibodies (Dako, Glostrup, Denmark) after staining with hematoxylin-eosin; normal lung samples were obtained from 3 age-matched subjects (3, 12 and 18 month-old) deceased from a non-pulmonary cause. Lung tissue was analyzed for amyloid by polarized light optical microscopy after Congo Red staining [13]. When frozen tissue was available, we

performed confocal fluorescence microscopy after dual immunolabeling with proSP-C, an endoplasmic reticulum (ER) marker (PDI) and a LB and lysosome marker (LAMP3) (see online supplement).

Transmission electron microscopy. Samples from cases #1-3 and 6 were obtained at the main institution following similar procedures, fixed in Karnovsky's fixative, postfixed in 1 % OsO4 and embedded in EMbed-812. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Zeiss EM CENTRA 100 microscope (Carl Zeiss, Oberkochen, Germany). Case #6 was obtained in another institution and processed differently: fresh bronchoalveolar lavage (BAL) samples were centrifuged at 800 RPM; cell pellets were resuspended in 2,5% glutaraldehyde in 0,1 M cacodylate buffer, postfixed in 1 % OsO4, dehydrated in graded ethanol and embedded in araldite. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips 410 microscope (Philips, Eindhoven, the Netherlands).

Results

Clinical features (table 1). All infants were Caucasian and of Italian origin except patient #4, who was of Romanian origin. They were born at term except patient #8 at 28 weeks of gestation, all with birth weight appropriate for gestational age (median 3,01 Kg; range 0.90-3.70), Four were males and four females. One had a family history of a sibling with fatal lung disease at 16 months. Median age at onset was 5.5 months (range 0-12). All except patient #8 showed significant postnatal failure to thrive. One infant (case #8) was a 28-week preterm triplet who presented with unexpectedly severe RDS compared to the two siblings, requiring multiple surfactant administrations, mechanical ventilation until 32 weeks, and

supplemental oxygen until 1 month corrected gestational age, whereas the other two siblings were only on CPAP for less than 3 weeks. The proband's subsequent course was normal, with a follow-up of 15 months. The other seven cases were asymptomatic in early infancy and presented with respiratory symptoms from 1-12 months, five of which after viral bronchiolitis. Lung high resolution computed tomography (HRCT) performed at a median age of 9 months (except case #7) showed ground glass opacities plus a combination of small and large cysts, subpleural nodules, lobar emphysema, air leaks and/or focal infiltrates. Case #7's prominent feature was multiple bronchiectasis [14]. Two infants died, at 6 and 8 months respectively; among survivors, two were on home oxygen therapy at 25 and 48 months with one enlisted for lung transplant, two were on room air with recurrent exacerbations requiring oxygen supplementation, and two were stable with no symptoms.

Molecular genetics. Of the 88 subjects of the cohort, eight harbored an *SFTPC* rare coding variant (table 2); in four (#2, 4, 5 and 6) the mutation was inherited from one parent, who was asymptomatic. Patient #1 carried the p.(Glu66Lys) mutation, previously reported to cause neonatal respiratory failure [15]; in addition, we found the unreported ABCA3 synonymous c.1383 G>A coding variant (p.(Val461=), LOVD:0000055959) of unclear clinical significance. Cases #2-5 carried the p.(Ile73Thr) mutation, the most frequently reported with a broad spectrum of manifestations from neonatal RDS to adult ILD [7,8,16-23]. Patient #6 carried the c.304G>A variation leading to the p.(Val102Met) aa change, previously reported without clinical description [13]. In patient #7, the c.463G>C variation was identified in exon 5, causing the p.(Ala155Pro) aa change, previously published as a case report [14]. Patient #8 carried the previously unreported c.518C>A variation leading to the p.(Pro173His) aa change. The new variants reported here were not present in 100

control alleles, and were neither found in 1000G nor in ESP except for patient #7 (c.463G>C variant, MAF 0.0359).

Anatomopathology. The four cases in whom a lung biopsy was performed (#1-3 and 6) showed a similar pattern of SP-B -positive alveolar epithelial type-II cells hyperplasia, alveolar spaces filled with SP-B -positive desquamated epithelial cells and alveolar macrophages, and interstitial thickening with fibroblast proliferation, corresponding to desquamative interstitial pneumonia (or pulmonary alveolar proteinosis in older literature).

Immunohistochemistry. In controls, immunostaining for proSP-C showed a focal cytoplasmic expression adjacent to LBs, which appear as clearer, outlined vesicles at the apical pole [9]. In subjects with linker domain mutations (cases #1, #2 and #3), there was increased staining for ProSP-C, which appeared to be localized largely to cytoplasmic vesicles similar to those identified by others as endosomes in the same p.(Glu66Lys) mutation [15]. In the BRICHOS mutant (case #6), there was also markedly increased proSP-C staining with some denser staining lining the nucleus, similar to the misfolded protein response pattern described in a Δexon4 (BRICHOS) mutagenesis experiment [24] (Figure 1).

Dual channel immunofluorescence. In the patient carrying the linker domain p.(Glu66Lys) mutation (case #1), confocal microscopy with co-labeling of SP-B and proSP-C showed a much stronger signal than SP-B compared to control, suggesting proSP-C overexpression (not shown). ProSP-C was only limitedly co-expressed with DPI in the p.(Glu66Lys) mutant as well as in the control lungs. However, the mutant showed a stronger

co-expression of proSP-C with LAMP3 than the control, with a pattern of large cytoplasmic vesicles (Figure S1, online supplement).

Polarized light microscopy. In cases #1 and 6 (Figure 2), small amyloid deposits with typical staining properties were identified in the interstitium, in association with macrophages, not found in lung tissue from control subjects. We attempted immunolabeling experiments with antibodies specifically targeted to the C-terminal propeptide and to the BRICHOS domain, but the small size of amyloid deposits precluded identification of immunoreactive material (not shown).

Transmission electron microscopy (figure 3). Compared to control, Cases #1, #2 and #3 (linker domain mutants) showed numerous , large organelles containing amorphous material and scarce electron-dense phospholipid structures likely corresponding to early endosomes [25]. Only a few MVBs and immature LBs with altered pseudomyelin structure could be observed in these subjects, the content of which is secreted in the alveolar lumen (case #1, figure S2, online supplement), suggesting a profound disruption of surfactant homeostasis. A similar pattern could be observed in an AEC2 isolated from bronchoalveolar lavage in patient #5, also a linker domain mutant. In contrast, patient #6 (BRICHOS mutant) showed a hyperplastic ER compared to control, perinuclear electron-dense cytoplasmic aggregates, abundant lysosomes, and some MVBs and immature LBs showing pseudomyelin structures, albeit less than in control.

Discussion

SP-C mutations are a significant and probably underestimated cause of diffuse lung disease

at various ages. Prevalence in pDLD series, as in ours, varies from 8 to 17% [1,3,6,7,19,26]. SP-C mutations have been described in term [15,27] or late preterm infants with severe RDS, although this presentation is more typical of SP-B and ABCA3 bi-allelic mutations [2]. The typical presentation of SP-C defects consists of dyspnea, cough or wheezing with an onset between 2 and 12 months of age, gradual cyanosis and failure to thrive [8,20,23,28]. In this paper the youngest subject, a 28-week preterm triplet carrying the p.(Pro173His) mutation was identified by comparing RDS severity with his two siblings with wild-type SP-C alleles. Conversely, in the literature, SP-C mutations have been found in adults with familial idiopathic pulmonary fibrosis [29] and in adults 14 to 68 year-old in one pedigree [30], suggesting that environmental factors or individual genetic background are significant determinants.

Steroids, hydroxychloroquine and azithromycin are commonly used in SP-C-related pDLD. Azithromycin has anti-amyloid properties [35], and hydroxychloroquine affects intracellular proSP-C processing [36]; hence these drugs may possibly have selective effects on specific mutations, although there are currently no clinical data available to support this concept. All of our subjects received one or more of these interventions at various points in their disease course, some before the biopsy was obtained, so it is difficult to know if these treatments modified the histologic or ultrastructural appearance in any fashion.

The common radiology characteristic in our cases was ground glass opacities, present in all cases except #7. Since median age at HRCT was 9 months except for case #7 (26 years), age may be a determining factor, although we cannot exclude mutation severity or other factors. In the literature, chest radiograms and/or HRCT show diffuse ground-glass opacities in

neonates[15], whereas in older infants and children, interstitial thickening associated with lung hyperinflation, intraparenchymal/supleural cysts, honeycombing, subpleural nodules [8,37], or bronchiectasis [14] are more prominent. These patterns are not different from those observed in other genetic surfactant deficiencies [38]. Infiltrates and air leaks are frequent complication in acute exacerbations [17]. Focal or diffuse reticulonodular patterns, centrolobular and subpleural nodules and cystic lesions are described in adults [29,30].

We did not observe specific differences in lung histology between our cases, in whom lung biopsy was obtained at a median age of 6 months. We observed a combination of DIP with features of PAP, a pattern common to most genetic surfactant deficiencies in infants [1]. In the literature, older children and adults with SP-C mutations display patchy fibrotic lesions of usual interstitial pneumonia or diffuse non-specific interstitial pneumonia, in combination with nodular septa thickening and clustered cystic lesions [29,30], suggesting lung tissue morphology is more age-specific rather than disease-specific.

However by TEM we observed significant anomalies in LB number and appearance in all cases examined, indicating altered surfactant composition/ structure. These alterations are distinct from those observed in SP-B or ABCA3 deficiency [3,6] and appear specific in both our observations and the literature [15,28]. In SP-C deficient mice, LBs and extracellular surfactant are similar to controls [39], suggesting that the human disease is not primarily caused by SP-C haploinsufficiency itself. However, even though human SP-C mutations are mono-allelic and both the wild type and the mutated alleles are transcribed, mature SP-C peptide is not (or barely) detected in lung tissue and bronchoalveolar lavage fluid [17,40]. Overall, these findings and data suggest that surfactant deficiency, due to a toxic gain-of-function mechanism

affecting lamellar body formation and surfactant synthesis, may contribute in part to the pathogenesis in human. In addition, in the 3 carriers of linker domain mutations in whom lung biopsy was performed (cases #1-3), we observed numerous large endosomes. Similar, albeit smaller, vesicles were observed in the other linker mutant (case #5), although the material was obtained by BAL and processed differently, hence may not be strictly comparable. Although very few TEM data have been published on human subjects with SP-C mutations, similar vesicles in one linker mutation case were shown by immunogold staining to co-localize with proSP-C, suggesting a trafficking defect [15]. Conversely in the single BRICHOS domain mutation carrier analyzed by TEM (case #6), we found hyperplastic ER and perinuclear electron-dense aggregates, which, if confirmed in other cases, could reflect misfolded protein-related cell toxicity [41].

Although bi-allelic mutations have been reported exceptionally [13,20], loss of function is not a prominent mechanism in human SP-C deficiency, which is typically caused by mono-allelic mutations. SP-C null mutant mice are viable, showing that SP-C is not critical for survival. Hence different pathophysiological processes, depending on distinct types of gene defects, are involved in human SP-C mutation-associated lung disease and, likely, account for this phenotypic heterogeneity.

Mutations in the linker (non-BRICHOS) sequence (aa59-89) of the C-terminal peptide exert their pathogenic effect by inducing aberrant intracellular trafficking of proSP-C, which eludes cleavage and accumulates in endosomes and MVBs [25]. In cases #1-3, we observed large proSP-C -positive vesicles in type II cell cytoplasm by immunohistochemistry. Confocal immunofluorescence microscopy in one case (#1) showed partial co-localization of proSP-C and LAMP-3, a marker expressed in MVBs and LBs, confirming abnormal

accumulation of the uncleaved pro-peptide in these organelles. Overall, our observations in human subjects are compatible with the model proSP-C misprocessing, accumulation in MVBs, aberrant secretion and reuptake in the endosomal/lysosomal pathway derived from animal and in-vitro studies [24,25] but are less clear-cut and may be of limited clinical use, likely because of the coexistence of a wild type and a mutated *SFTPC* allele, both expressed to a certain degree and resulting in a mixed phenotype.

The majority of mutations reported in human affect the highly conserved BRICHOS domain (aa90-197), common to proSP-C and other proteins associated with dementia, and bone and gastric cancer. The mature SP-C peptide is highly hydrophobic and tends to form β-sheets aggregates and amyloid fibrils in vitro [13,42]. In fact, overexpression of the mature SP-C peptide in transgenic mice leads to neonatal lethality[42], The BRICHOS domain functions as a chaperone, preventing aggregation and amyloid formation. In-vitro mutagenesis and transgenic mice studies demonstrated that BRICHOS domain mutations lead to SP-C misfolding, accumulation in the ER, amyloid formation, disruption of the ubiquitin-proteasome system, activation of the apoptosis cascade, and type II cell death. [13,24,33,43]. The proSP-C perinuclear expression pattern observed in case #6 may reflect peptide segregation and aggregation in the ER, but confirmatory confocal immunofluorescence studies with a specific ER marker could not be performed in this case due to the lack of frozen tissue, and more cases would be necessary to confirm these speculations. We could demonstrate the presence of amyloid in the interstitium in one of the four subjects with linker domain mutations, further supporting this mechanism. However, amyloid was also detected in patient #1, suggesting that protein misfolding occurs to some degree in linker domain mutants.

However, these conclusion should be balanced by the limited number of cases with lung

tissue available, and by the fact that, as in most pathology studies based on human cases, it is difficult to establish which findings are part of the underlying disease process per se, which are a response to the underlying mechanism of disease, and which might be the result of interventions (such as mechanical ventilation) or duration or severity of disease.

The 5 subjects with linker domain mutations carried known pathogenic variants, 4 of which were p.(Ile73Thr). Although linker domain mutations have been reported to have a less severe clinical expression than BRICHOS mutation in some series [7,17], our own data and the literature do not necessarily support this concept. We found three BRICHOS domain rare variants in our series. No other *SFTPB*, *ABCA3* or *NKX2.1* mutations were present in these subjects. The p.(Val102Met) variant (case #6) is predicted to be deleterious in silico by both algorithms and is mostly conserved among species; its pathogenic role is supported by histologic and ultrastructural findings including amyloid deposits [13]; it was also reported in another patient with neonatal-onset respiratory failure [16, online supplement]. The p.(Phe173His) variant (case #8), unreported so far, appears damaging on PolyPhen-2 but not on SIFT, and is also less conserved among species (table 2), making its pathogenicity questionable. The more severe clinical course in the proband compared to the non-mutated siblings supports mutation pathogenicity, but prematurity (as a potential cause of lung disease) and the favorable outcome may suggest it as a benign variant. The role of the p.(Arg155Pro) variant (case #7) is also less clear, as this missense mutation shows a low damaging probability and has been reported in three subjects of European descent (0.04%) in the NHBLI Exome sequencing project. Since no lung tissue was available for phenotype correlation and the parents did not consent for genetic testing, we cannot affirm that this variant is responsible for the subject's lung disease. The relevance of the associated

synonymous ABCA3 mutation observed in patient #1 is uncertain. Compound genotypes associating I73T and an ABCA3 mutation have been reported in infants with pDLD [19] and in adults with late-onset ILD, suggesting that a mono-allelic ABCA3 mutation may act as disease modifier in SP-C defects [21].

Conclusion

This case series illustrates the vast array of symptoms and outcomes associated with SP-C deficiency, and shows correlations between known and new genotypes and lung ultrastructure, supporting the importance of lung biopsy including TEM analysis for accurate diagnosis. Broncho-alveolar lavage may represent an alternative, less invasive diagnostic procedure than lung biopsy if validated in future studies. Since this clinical variability is only partially supported by molecular and genetic mechanisms, future research should focus on determining individual genotypes through genomic approaches, in order to expand the understanding of genetic-clinical correlations and interactions with other surfactant-related genes.

This study was limited by the number of cases and by the lack of mutagenesis studies susceptible to better establish disease mechanism correlations. As for any rare disorder, only large collaborative initiatives may yield the sufficient power of validating new diagnostic and therapeutic approaches.

Acknowledgements

We are grateful to Dr. Paola Carrera, Clinical Molecular Biology Laboratory, Università Vita-Salute San Raffele, Milan, Italy for *SFTPC* sequencing in patient #5. This publication was

supported by an unrestricted grant by the Chiesi Foundation, Parma, Italy

Conflict of interest statement.

The authors declare that there is no conflict of interests regarding the publication of this paper. The studies were conducted in compliance with the hospitals internal review boards and research committees.

Supplementary information is available at European Journal of Human Genetics's website (http://www.nature.com/ejhg).

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Titles and legends to figures

Figure 1: Lung tissue morphology and surfactant protein immunohistochemistry. Immunohistochemistry staining for SP-B (columns 1 and 2, 10x and 40x magnification respectively) and pro-SP-C (columns 3 and 4) in a 3-month-old control (row 1), three linker domain mutants (row 2-4) and one BRICHOS SP-C mutant (row 5). AEC2 hyperplasia is present in all four cases compared to control. In control, proSP-C is expressed in the cytoplasm surrounding clearer-appearing lamellar bodies (row 1, insert, arrows). In cases #1-3, proSP-C is diffusely overexpressed in the cytoplasm with a granular pattern (row 3, insert, arrows). In case #6, proSP-C is overexpressed with a perinuclear pattern and some scattered aggregates (row 5, insert, arrows). Counterstaining with hematoxylin-eosin.

Figure 2: Amyloid detection. A. Congo red staining of case #6 lung specimen showing a small Congo-positive deposit in the interstitium, near cholesterol clefts (arrow). B. Same specimen viewed under polarized light optical microscopy, the deposit showing green birefringence indicating amyloid (arrow).

Figure 3: Type II cell ultrastructure. Type II cell sections in a control subject, four linker domain mutation carriers (cases #1-3 and #5) and one BRICHOS mutation carrier (#6). Tissue was obtained by autopsy for control and live biopsy for cases #1-3 and 6; cells were obtained by bronchoalveolar lavage for case #5. In control, numerous mature lamellar bodies with pseudomyelin are present (arrow). Cases #1-4 show numerous, large coalescing endosomes with scarce amorphous content (arrow), some lysosomes, and few multivesicular bodies (inserts). Case #6 shows hypertrophic endoplasmic

reticulum (*) compared to control, some cytoplasmic electron-dense deposits (block arrows) and several multivesicular bodies (arrows) with disorganized phospholipid membranes and amorphous content (insert).

Table 1. Clinical course and phenotype.

Case	Sex, BW, GA, family history	Presentation and clinical course	HRCT imaging	Treatment and outcome	ProSP-C expression and amyloid staining	Ultrastructure
1	F; term; 3,30 Kg Parents and one sibling healthy	Dyspnea and failure to thrive at 1 m PICU from 3 m Lung biopsy 3 m	3 m: ground glass opacities, upper lobes cysts, upper lobar emphysema, PTX	O_2 from 1 m, MV from 3 m PS + HCQ from 3 m Death 6 m	AEC2 hyperplasia Granular proSP-C pattern with diffuse large aggregates Several amyloid deposits	Many large endosomes Very rare LBs with abnormal PL structure
2	M; term; 3,70 Kg Parents healthy, no siblings	Multiple bronchiolitis episodes 3-12 m Hypoxemia from 14 m PICU from 14 m Lung biopsy 18 m	14 m: ground glass opacities, multifocal interstitial infiltrates	O_2 from 14 m, MV from 15m, tracheostomy 16 m PS + HCQ from 15 m Death 19 m	Marked AEC2 hyperplasia Granular proSP-C pattern with diffuse large aggregates No amyloid detected	Many large endosomes with some PL content Very rare immature LBs and MVBs
3	M; term; 3.00 Kg Healthy parents and sister	Bronchiolitis 5 m PICU 9-13 month for respiratory failure, recurrent PTX Lung biopsy 15 m	9 m: ground glass opacities, multiple cysts	O_2 from 11m, MV 9-11 m PS + HCQ from 13 m Alive on O^2 at 48 m Listed for transplant	AEC2 hyperplasia Granular proSP-C pattern with diffuse large aggregates No amyloid detected	Many large endosomes with PL content Very rare LBs
4	F; term; 3.30 Kg 1 sibling, fatal respiratory failure 16 m	Chronic cough and dyspnea since 7 m PICU 18-19 m for hypoxemia and dyspnea	10 m: Diffuse ground glass opacities, honeycombing, multiple subpleural nodules.	O_2 18-21 m HCQ from 21 m Alive on room air at 30 m	n/a	n/a
5	M, Term, 2.990 Kg Parents healthy, no siblings	Chronic cough, failure to thrive since 9 m Hospitalized 13-14 m for dyspnea and hypoxemia	13 m: Diffuse ground-glass opacities	O_2 13-19 m Steroids for 5 m at 13 m AZM 18 m, HCQ from 13 m Alive on room air at 21 m	n/a	(tracheal aspirate) Some large endosomes with PL content Rare MVBs
6	F; term; 3.03 Kg Parents healthy, no siblings	Hospitalized for bronchiolitis at 6m Hospitalized 11-15 m for hypoxemia and PTX, lung biopsy at 14 m	6 m: diffuse ground glass opacities, basal emphysema	O_2 since 13 m, MV 6-7 m PS 11-12 m HCQ from 16 m Alive on O^2 at 24 m	Marked AEC2 hyperplasia Some granular proSP-C pattern plus perinuclear aggregates Rare amyloid deposits	Numerous mitochondria, lysosomes and electron-dense deposits Several normal LBs Several MVBs

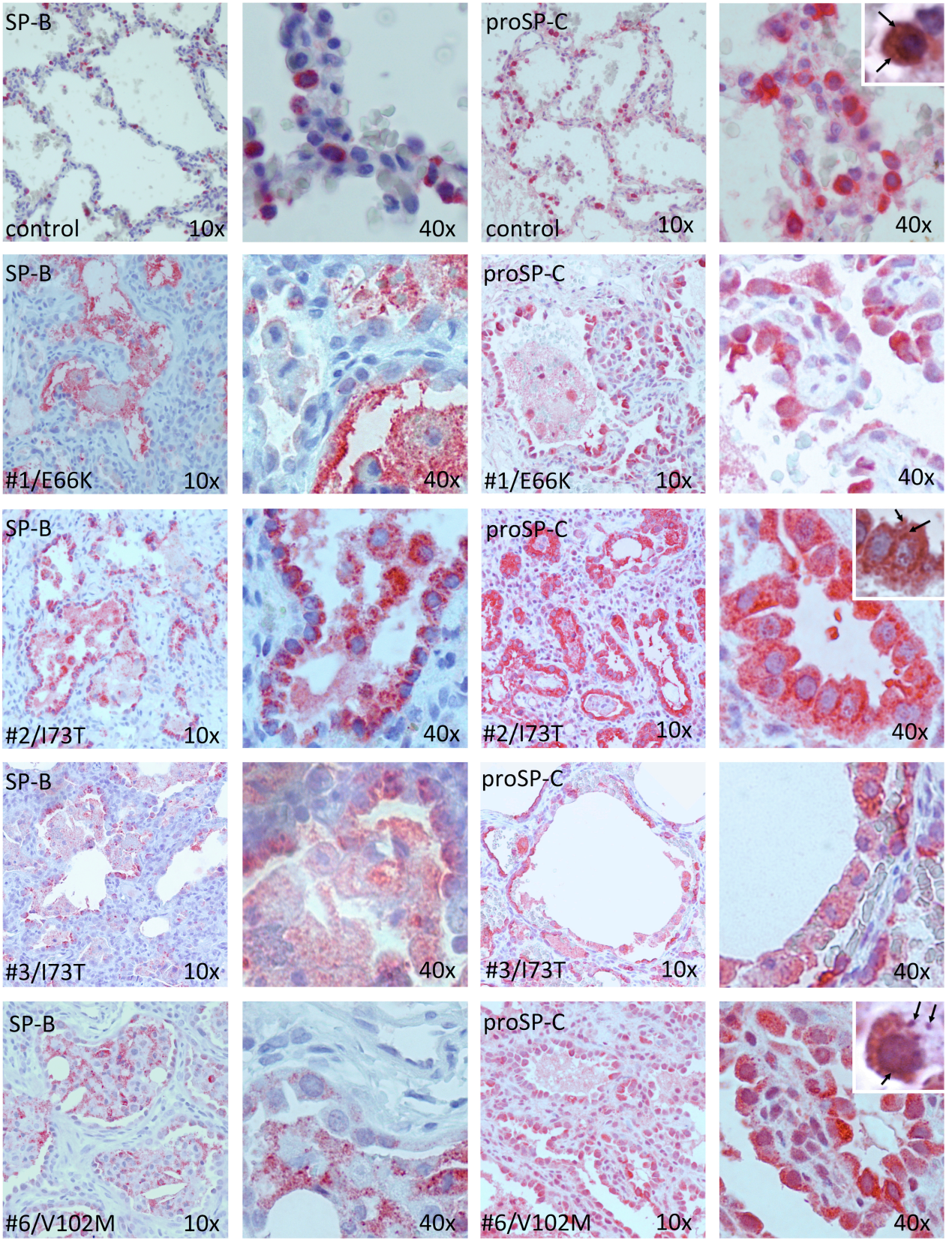
7	F; term; 3.00 Kg Parents healthy, no siblings	Pneumonia at 1 y, Several hospitalizations for LRTI in childhood; Severe failure to thrive Hypoxemia at 26 y	26 y: mild interstitial lung disease, diffuse tubular and varicose bronchiectasis, basal infiltrates	Intermittently on O_2, never ventilated Multiple antibiotic and PS courses, bronchodilators Alive at 28 y on O_2	n/a	n/a
8	M; 28w; 0.90 Kg Parents healthy, two triplet siblings with no CLD	Severe RDS at birth, 2 doses of surfactant, MV until 4 w	2 m: diffuse ground-glass opacities, overexpansion, bronchial markings	O_2 2-4m, MV 1 m Steroids 1 m Alive on rom air at 14 m	n/a	n/a

Abbreviations:
M: male; F: female; w: weeks; m: months; y: years; RDS: respiratory distress syndrome; CLD: chronic lung disease; m: month; Y: year; PICU: pediatric intensive care unit; PTX: pneumothorax; LRTI: lower respiratory tract infection; MV: mechanical ventilation; XR: radiogram; CT: computed tomography scan; ILD: interstitial lung disease; O_2: supplemental oxygen; PS: pulse steroids; HCQ: hydroxychloroquine; AZM: azithromycin.

Table 2. SFPTC Variants Found in Individuals

Case	Variant[a]	Inheritance	dbSNP/LOVD	Annotation	SIFT/Polyphen2	1000G/ESP[b]	Species conservation
1	**c.196G>A p.(E66K)**	Paternal	rs121917836 0000053109	Pathogenic[c]	Tolerated, score 0.18/probably damaging, 0.99	nd/nd[d]	Highly conserved
2	**c.218T>C p.(I73T)**	Paternal	rs121917834 0000053110	Pathogenic[e]	Damaging, score 0/possibly damaging, 0.85	nd/nd	Mostly conserved
3	**c.218T>C p.(I73T)**	Paternal	rs121917834 0000053110	Pathogenic	Damaging, score 0/possibly damaging, 0.85	nd/nd	Mostly conserved
4	**c.218T>C p.(I73T)**	Unknown; sibling died of RDS	rs121917834 0000053110	Pathogenic	Damaging, score 0/possibly damaging, 0.85	nd/nd	Mostly conserved
5	**c.218T>C p.(I73T)**	Maternal	rs121917834 0000053110	Pathogenic	Damaging, score 0/possibly damaging, 0.85	nd/nd	Mostly conserved
6	**c.304G>A p.(V102M)**	Sporadic	[g]See footnote 0000053108	nd	Tolerated, score 0.08/probably damaging, 1.00	nd/nd	Highly conserved
7	c.463G>C p.(A155P)	Unknown	rs202145169[h] 0000053106	nd	Tolerated, score 0.28/Benign, 0.004	nd/0.0359 EA[i]	Variable
8	c.518C>A p.(P173H)	Sporadic 2 siblings negative	Not reported 0000053104	nd	Tolerated, score 0.08/possibly damaging, 0.94	nd/nd	Variable

[a]Column 2: in bold, established or probable disease-causing variants; in light, variants of uncertain significance.
[b]1000 Genomes Project phase1, NHBLI Exome sequencing project
[c]OMIM, http://www.ncbi.nlm.nih.gov/pubmed/15557112
[d]nd=no data, not present
[e]OMIM, http://www.ncbi.nlm.nih.gov/pubmed/11991887, http://www.ncbi.nlm.nih.gov/pubmed/15293602, http://www.ncbi.nlm.nih.gov/pubmed/19443464
[f]synonymous variant
[g]Willander et al. *Proc Natl Acad Sci U S A*, vol. 109, no. 7, pp. 2325-2329, 2012, online supplement.
[h]Reported in three European Americans in ESP, heterozygous
[i]Minor allele frequency in European Americans in ESP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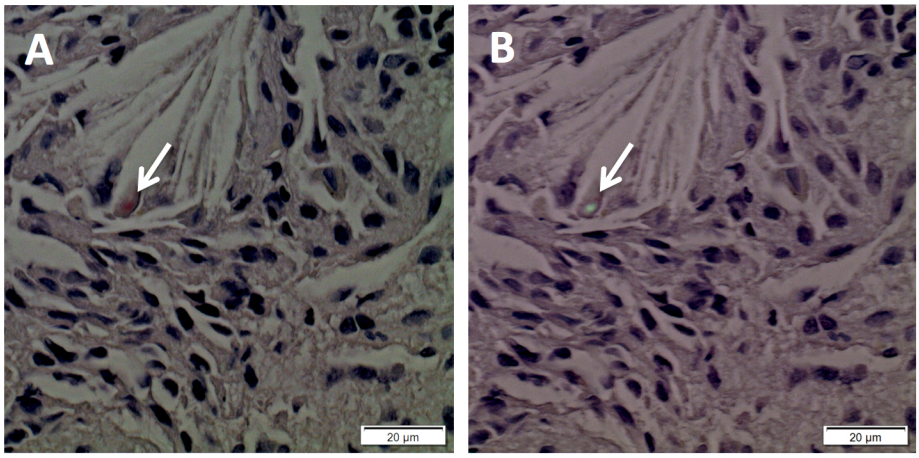
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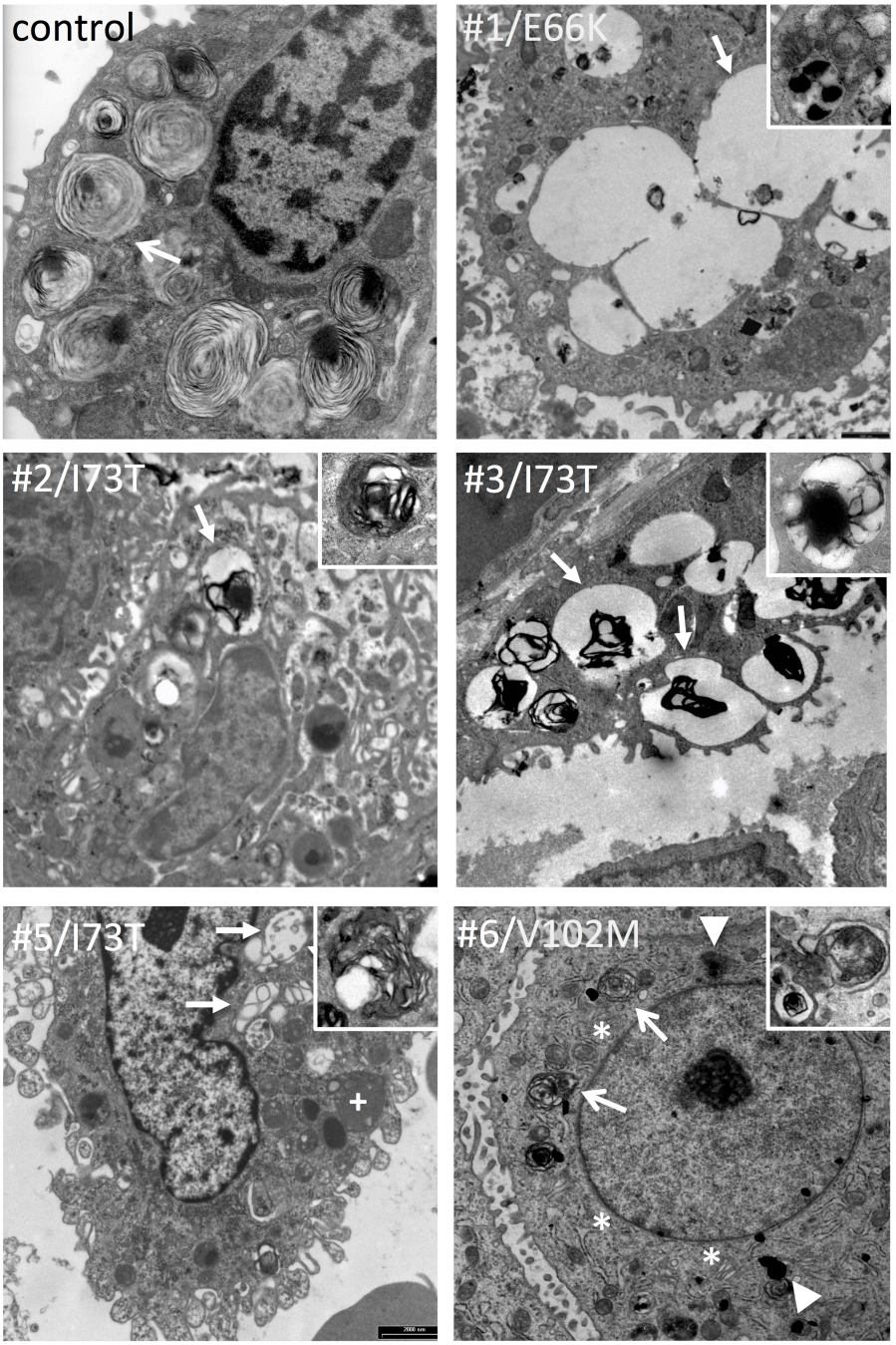
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#5/I73T
#6/V102M

Supplementary material
Supplemental data and methods
Figure S1 and S2

Clinical and ultrastructural spectrum of diffuse lung disease associated with surfactant protein C mutations

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Running title:
Histopathology and ultrastructural features of SP-C mutations

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Diffuse lung disease cohort characteristics

From January 1st 2005 to June 30th 2012, 121 children 0-18 year-old were evaluated for diffuse lung disease. After exclusion of active bacterial or viral infection including tuberculosis and aspergillosis, immune deficiencies, primary ciliary dyskinesia, chronic tracheal aspiration, congenital heart disease with left-to-right shunt, cystic fibrosis and other causes, genetic testing was performed, with specific gene selection based on age of onset and clinical presentation. For neonates with fatal hypoxic respiratory failure, *SFTPB* and *ABCA3* were tested first; for those with severe pulmonary hypertension suggesting alveolar capillary dysplasia, *FOXF1* (NM_001451.2) was added. In later-onset cases *SFTPC* was tested primarily, then *ABCA3* when the former was negative. *NKX2.1* (NM_001079668.2) was primarily tested in cases with associated hypothyroidism or movement disorder. Additional testing was performed in cases that remained unexplained. The number of patients tested for these genes and the relative proportion of mutants detected is indicated in the following table:

Total (n)	121		
cases tested (n)		bi-allelic mutations	mono-allelic mutations
SFTPB	49	0	3 (6%)
SFTPC	88	0	8 (9%)
ABCA3	74	10 (14%)	4 (5%)
NKX2.1	16	0	2 (12%)
FOXF1	15	0	2 (13%)

Other congenital diseases were found in six cases: pulmonary interstitial glycogenosis in two, cytochrome oxidase deficiency in one, Niemann-Pick disease type B in one, alveolar capillary dysplasia without *FOXF1* mutation in two.

Confocal microscopy methods: studies were performed in one patient (case #1), in whom frozen lung tissue was available, and in an age-matched control subject. Serial lung cryosections (5 μm) were air-dried and incubated with polyclonal anti proSP-C (Millipore, Billerica, MA) diluted in 0,1 M phosphate buffered saline (PBS, pH 7.4) containing 1% bovine serum albumine (BSA). After PBS washes, slides were incubated with goat anti rabbit Alexa Fluor 488-conjugated immunoglobulin (Molecular Probes, Eugene, OR). Double staining was performed using one of the following mouse monoclonal antibodies: anti SP-B (Labvision, Fremont, CA), protein disulphide isomerase (PDI, Molecular Probes, Eugene, OR), and LAMP3 (Santa Cruz Biotechnology, Santa Cruz, CA) and revealed with goat anti-mouse IgG conjugated with Alexa Fluor 555 (Molecular Probes, Eugene, OR). Negative controls were performed using PBS/BSA 1% without the primary antibody. Slides were mounted in 50% glycerol in PBS and examined using an Olympus IX81 inverted microscope equipped with epifluorescence optics; image acquisition and co-localization analysis were performed using an Olympus fluoview FV1000 confocal microscope equipped with FV10-ASW version 2.0 software, Multi Ar (458-488 and 514 nm) and 2X He/Ne (543 and 633 nm) lasers with 60X oil immersion objective (Olympus, Center Valley, PA). Images were processed using Adobe Photoshop 9.0 software (Adobe, San Jose, CA).

Figure S1 legend

Confocal microscopy analysis after immunofluorescent labeling of proSP-C in green, and either protein disulphide isomerase – an endoplasmic reticulum marker - (PDI) or lysosome-associated membrane protein 3 (LAMP3), a multivesicular and lamellar body marker, both in red; co-expression pattern shown in merged images (column 3). In control (line 1 and 2), proSP-C shows limited co-localization with PDI and LAMP-3. In contrast, in the p.(Glu66Lys) mutation carrier (case #1) limited proSP-C co-localization with PDI is observed, but there is a stronger proSP-C colocalization with LAMP3.

Figure S2 legend

Transmission electron microscopy of lung tissue in patient #1, showing an alveolar epithelial type 2 cell secreting large vesicles with heterogeneous content in the alveolar lumen.

Figure S1

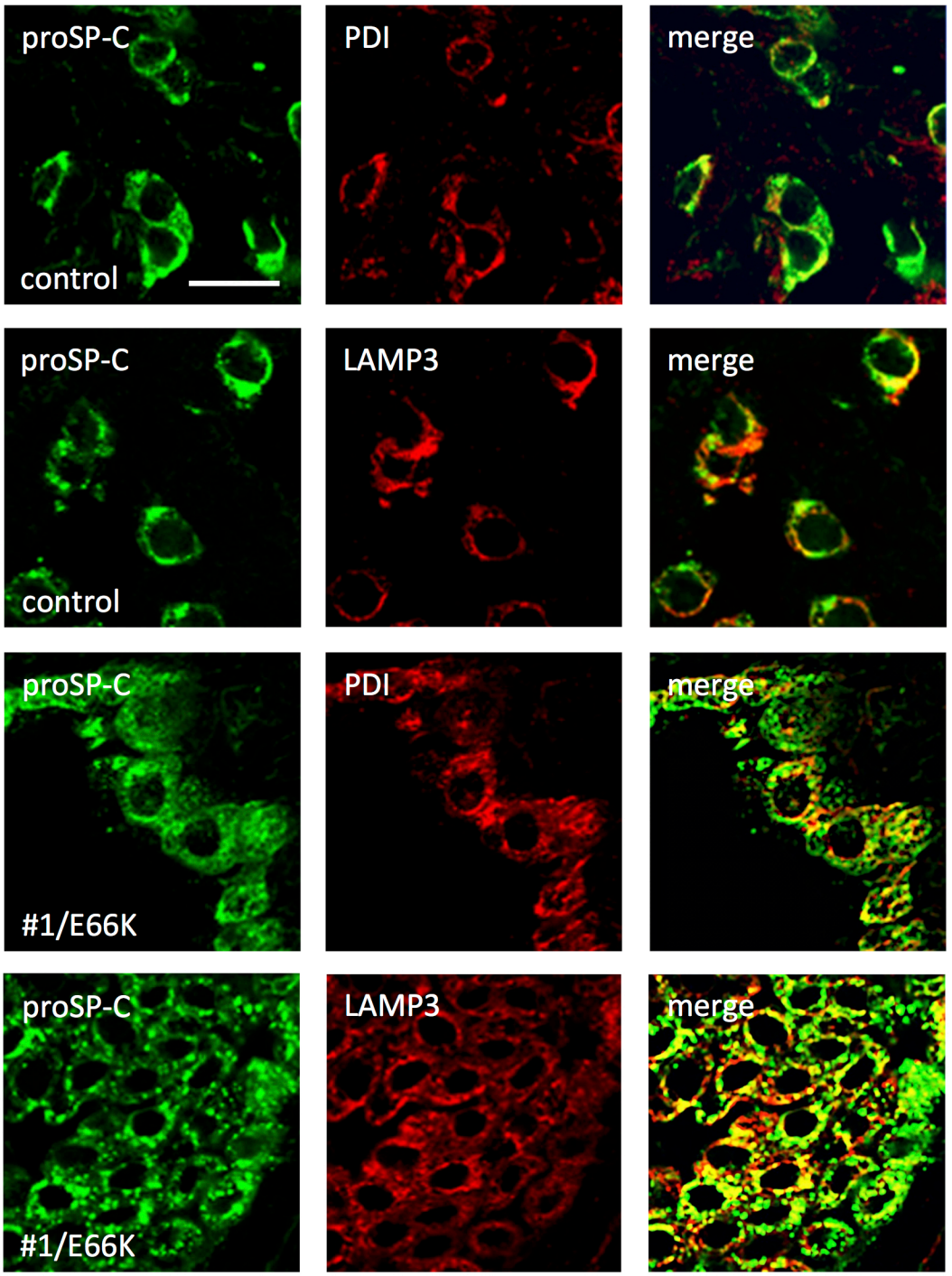
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Figure S2