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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Electron Tomographic Analysis of Dystonia Pathogenesis

A thesis submitted in partial satisfaction of the
Requirements for the degree Master of Science

in

Bioengineering

by

Alexander Joseph Perez

Committee in charge:

Professor Mark H. Ellisman, Chair
Professor Geert W. Schmid-Schönbein
Professor Kun Zhang

2008

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University of California, San Diego

2008

DEDICATION

This thesis is dedicated to my family – my parents, sister, and grandfather – for your unconditional support throughout my education and life. Thank you for teaching me, believing in me, and inspiring me to pursue my goals.

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LIST OF ABBREVIATIONS

AAA	ATPase associated with different cellular activities
ATP	adenosine triphosphate
BTX	botulinum toxin
CNS	central nervous system
DYT	dystonia type
EMG	electromyography, electromyographic
ER	endoplasmic reticulum
ET	electron tomography
GAG	guanine-adenine-guanine
GPI	globus pallidus internus
GUI	graphical user interface
HEK	human embryonic kidney
IBS	inner bleb structure
INM	inner nuclear membrane
KO	knockout
LAP	lamina-associated peptide
LSR	luminal spoke ring
LULL	luminal domain like LAP1
NE	nuclear envelope
NPC	nuclear pore complex
NR	nuclear ring
ONM	outer nuclear membrane
PET	positron emission tomography
POM	pore membrane
ROI	region of interest
SD	standard deviation
SMC	supplementary motor cortex
SPA	single-particle analysis
S-V	surface area to volume
TEM	transmission electron microscope
TxBR	transform based back projection
VMAT2	vesicular monoamine transporter 2
WT	wildtype

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An adaptation of the text and figures in Chapters 2, 3, and 4 will be submitted for publication with the following co-authors: Guy Perkins, Mark Ellisman, William Dauer, Guido Gaietta, and Tom Deerinck.

ABSTRACT OF THE THESIS

Electron Tomographic Analysis of Dystonia Pathogenesis

by

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Master of Science in Bioengineering

University of California, San Diego, 2008

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The dystonias are a set of movement disorders associated with neural dysfunction. Due to the large variety in etiology, symptoms, and age of onset of the dystonias, relatively little is known about their pathogenesis and treatments are often administered on a trial-and-error basis. A majority of the cases of DYT1 dystonia, a severe early-onset variant of the disease, are caused by the deletion of a single

amino acid in torsinA, a AAA+ protein. The mutation causes a redistribution of torsinA to the nuclear envelope where it acts on LAP1, a protein of the nuclear lamina. The torsinA mutation manifests itself physically by causing the formation of nuclear envelope blebs and perinuclear vesicles. Electron tomography, a tool for producing three-dimensional reconstructions of sub-cellular structures, was used to explore the ultrastructure of these physical abnormalities. The evidence suggests that blebs bud from a single nuclear pore, remain temporarily attached to the inner nuclear membrane via a membranous neck, and eventually bud off into the perinuclear space as vesicles. This process was the same in both torsinA and LAP1 knockout cells, indicating that a normal interaction between the two is necessary to prevent vesicle formation. Nuclear pore density was dramatically decreased from expected levels, indicating that pore damage occurs concomitantly with bleb formation. A set of novel structures, named inner bleb structures, were found to be specific to torsinA KO neurons. Taken together, these findings help elucidate the relatively unexplored pathway of dystonia pathogenesis and may lead to more effective and targeted treatments.

CHAPTER 1: BACKGROUND & INTRODUCTION

1.1. Dystonia

The dystonias are a broad set of movement disorders associated with neurologic dysfunction (Crossman and Brothie, 1998). Although the clinical manifestations of the disease vary on a patient-by-patient basis, abnormalities often include uncontrollable twitching, repetitive movements, and abnormal postures (Fahn et al., 1998). These conditions are caused by involuntary muscle contractions that may be sustained, spasmodic, patterned, or repetitive, and are often exacerbated by voluntary movements (Brin and Comella, 2004). Patients afflicted with dystonia typically suffer from continuous pain, cramping, and muscle spasms as a result of the perpetual contractions. The majority of dystonic movements have an associated directionality, a fact that sets them apart from other neurologic diseases such as tremor.

1.1.1. Classification & Epidemiology

Clinically, patients with dystonia are described according to three categories: age of onset, symptom distribution, and etiology (Fahn et al., 1998; Klein and Ozelius, 2002). Etiologically, the dystonias are placed into two broad categories based upon the presumed origin of disease. The secondary, or symptomatic, dystonias develop as a result of environmental factors or accidents that cause trauma to the brain. All other syndromes for which dystonia is the only phenotypic manifestation are considered primary dystonias. The primary dystonias may occur as the result of familial inheritance or may be sporadic and without a discernible hereditary pattern.

The age of onset of the first dystonic event is a critical indicator of the likelihood that this single, localized event will spread to other body parts. Age of onset is divided into three categories: infantile onset (<2 years old), childhood onset (2-26 years old), and adult onset (>26 years old) (Brin and Comella, 2004). Infantile-onset dystonias are extremely rare and are typically the result of metabolic dysfunction, trauma, or other secondary causes. The majority of primary dystonias fall into the childhood, or early, onset category. The distribution of dystonic symptoms may be localized to a specific body region (focal), spread across multiple contiguous body regions (segmental), or spread across multiple non-contiguous body regions (generalized).

Although extensive epidemiological studies of dystonia are limited due to the broad scope and misdiagnosis of the disease, a few studies have been carried out to give estimates of its extent. Surveys of primary dystonia conducted in Europe and Japan yielded prevalence rates of 152 and 101 per million, respectively (Group, 2000; Matsumoto et al., 2003). Due to misdiagnosis and the fact that many patients suffering from dystonia fail to seek medical attention, these numbers are thought to

Table 1.1: Overview of the categories used clinically to classify the dystonias.

Age of Onset	
Juvenile	< 2 years old, rare, generally due to trauma or metabolic dysfunction
Childhood	2-26 years old, majority of dystonias fall in this category
Adult	>26 years old
Symptom Distribution	
Focal	Affects only one body region, typically a limb
Segmental	Symptoms spread across multiple contiguous body regions
Generalized	Spread across multiple non-contiguous body regions or whole body
Etiology	
Primary	Genetic or idiopathic (etiology is unknown)
Secondary	Caused by accident or other insult (environmental, metabolic)

be underestimates. One study approximates that an additional 111 people per million suffer from dystonia but are misdiagnosed or fail to seek treatment (Defazio et al., 2004). Although dystonia is considered to be a rare disease, it is believed that the total prevalence of all forms of dystonia is about 390 per million, a number that makes dystonia more widespread than well-known diseases such as muscular dystrophy and Huntington's disease (Marsden and Quinn, 1990).

1.1.2. Dystonia Pathogenesis

The physiological changes that lead to dystonia are not well understood, but a variety of studies have begun to elucidate possible mechanisms. It has long been known that dystonic movements are characterized by abnormal electromyographic (EMG) activity with co-contraction of antagonist muscles (Cohen and Hallett, 1988). EMG bursts resulting from quick movements last between 200 and 300 milliseconds in dystonic patients compared to the 100 millisecond duration typically seen in non-dystonic individuals (Hallett, 1998). A variety of evidence has led to the belief that basal ganglia dysfunction and altered signaling through the basal ganglia may be the key physiological abnormalities in dystonic patients (Bhatia and Marsden, 1994; Marsden et al., 1985).

Although basal ganglia dysfunction has been heavily implicated as a cause for dystonic movements, the mechanism of its influence is currently not well understood. The basal ganglia directly influence the motor system via the globus pallidus internus (GPi), the main outflow nucleus of the basal ganglia. The GPi provides functional inhibition to the thalamus, which in turn stimulates the supplementary motor cortex (SMC). It follows that altered GPi activity may cause distorted patterns and

abnormalities in the motor system. A positron emission tomography (PET) study analyzing the uptake of a radiolabeled dopamine analog, [^{18}F]dopa, found a moderate reduction in uptake in the putamen of the basal ganglia of dystonic patients (Playford et al., 1993). Under the current model of basal ganglia activity, reduced dopamine input into the motor pathways results in increased GPi activity (Ondo, 2004). However, the GPi firing rate of dystonic patients is found to be significantly decreased from baseline levels, with a highly irregular firing pattern (Sanghera et al., 2003). In addition, motor cortex firing rate may be either increased or decreased depending on the class of dystonia. Due to these complications and the dynamic and diverse nature of dystonia, an exact pathophysiological mechanism to explain dystonic movements has remained elusive.

1.1.3. Current Outlook & Treatments

Though dystonia is rarely fatal, currently practiced medical treatments are aimed at controlling symptoms rather than treating the cause and are often ineffective or difficult to administer. The course of dystonia therapy is determined by a variety of factors, including patient age, category of dystonia, and symptoms. Segmental and generalized dystonias are typically treated with a variety of oral medications, including levodopa, Baclofen, tetrabenazine, and anticholinergics (Bhidayasiri, 2006). However, patient response to these drugs varies, and drugs are often administered in a trial-and-error fashion to establish effectiveness. Focal dystonias are generally treated using local injections of botulinum toxin (BTX) into the affected area. BTX inhibits acetylcholine release at the neuromuscular junction, leading to temporary muscle paralysis and relief from uncontrollable contractions. In cases where drug

administered treatments are not responsive, a variety of surgical options may be employed, including deep brain stimulation of the GPi to disrupt abnormal GPi discharge patterns (Jankovic, 2006). Although these treatments present diverse options for dystonic patients, they are hardly perfect and most are administered on a trial-and-error basis due to their highly unpredictable responses. To develop more effective and consistent treatments, a better understanding of dystonia pathogenesis is necessary.

1.1.4. DYT1 Dystonia: Overview & Genetics

DYT1 dystonia is the most severe form of the 14 hereditary dystonias for which chromosomal locations are known. DYT1 usually falls into the early onset category, with the first dystonic symptom typically beginning around the age of twelve (Augood et al., 1998). The first focal dystonic event almost always occurs in a limb, with 90% of first events occurring in either an arm or leg (Fahn et al., 1998). After a characteristic duration of five years, the focal event spreads and becomes generalized, affecting various other body parts. It is this proliferation and variety that make DYT1 dystonia such a difficult disorder to treat.

The chromosomal location of the gene locus responsible for DYT1 dystonia was discovered in 1989 (Ozelius et al., 1989). The DYT1 (TOR1A) gene is located at human chromosomal position 9q34, and has been identified to code for the protein torsinA (Ozelius et al., 1997). A single 3 base pair deletion (Δ GAG) in the coding region of TOR1A leads to the removal of a single codon and subsequent loss of a glutamic acid residue (Δ E) from the carboxy terminal of torsinA. The mutation is autosomal dominant in its inheritance and displays a reduced penetrance of about

30%. Genetic screening has concluded that this single mutation is the sole cause of disease in 50-60% of individuals diagnosed with DYT1 dystonia (Bressman et al., 1994). Screens for other mutations in DYT1 patients were unsuccessful, suggesting that the changes in torsinA caused by the Δ GAG deletion may be unique. In an attempt to understand the mechanism behind DYT1, the focus of the following section is shifted to the function and pathology of torsinA.

1.2. TorsinA & the Nuclear Envelope

1.2.1. *The Nuclear Envelope*

Since many aspects of torsinA function and pathology involve the nuclear envelope (NE), it is prudent to begin with an overview of its structure and function. The NE is a specialized subdomain of the ER and is composed of a set of two concentric membranes, the inner nuclear membrane (INM) and outer nuclear membrane (ONM). The INM and ONM are joined at nuclear pore complexes (NPC) that are scattered throughout the NE. At NPCs, the INM and ONM are fused together to form what is referred to as the pore membrane (POM). The lumen between the INM and ONM, the perinuclear space, is continuous with the lumen of peripheral ER and has a thickness of about 50 nm (Prunuske and Ullman, 2006). The INM, ONM, and POM are each associated with a set of distinct proteins. The ONM is associated with the cytoskeletal actin network while the INM is lined by the nuclear lamina, a 20-50 nm protein mesh essential for maintaining the structural integrity of the NE (Burke and Stewart 2002). The NE controls movement of macromolecules between the nucleus and cytoplasm and supplies a site for the anchoring of chromosomes (Gerace, 2004). A schematic drawing of the NE can be seen in Figure 1.1.

1.2.2. TorsinA

Recent research has shed light on the structure and function of torsinA, the protein coded for by the TOR1A gene locus. TorsinA bears a significant homology to the Hsp100/Clp family of ATPase chaperones and belongs to the family of AAA+ (ATPases associated with a variety of cellular activities) proteins (Kustedjo et al., 2000). The ATPases are a family of proteins with a conserved AAA domain that catalyze ATP hydrolysis to mediate conformational changes in target proteins (Vale, 2000). TorsinA is ubiquitously expressed in many tissues of the human body, with the highest level of expression in the liver (Ozelius et al., 1997). In the brain, expression of torsinA is greatest in neurons located in the cortex, hippocampus, and basal ganglia, as well as dopaminergic neurons in the substantia nigra (Augood et al., 1999). Wildtype (WT) torsinA is located predominantly in the lumen of the ER (Goodchild and Dauer, 2004). Three proteins homologous to torsinA have been identified: torsinB, torp2a, and torp3a. Related coding sequences are found in various eukaryotic organisms but not in prokaryotic ones, suggesting an evolutionary importance of the torsins in sustaining multicellular life (Breakefield et al., 2001).

1.2.3. The Abnormal Behavior of Mutant TorsinA

The Δ GAG in-frame mutation of torsinA that is responsible for the majority of cases of DYT1 dystonia results in abnormal cellular torsinA function and chemistry. This mutation has been shown to cause a dramatic relocalization of torsinA from the ER to the NE in both neuronal and non-neuronal cells (Goodchild and Dauer, 2004). Mutant torsinA, Δ E-torsinA, is concentrated within the perinuclear space of the NE where it is hypothesized to interact with the luminal region of a NE protein. To test

this hypothesis, an acidic residue in the torsinA Walker B motif essential for ATP hydrolysis was removed, essentially locking bound ATP to the protein (Goodchild and Dauer, 2004; Naismith et al., 2004). This removal caused a large accumulation of ATP-bound torsinA in the NE, indicating that torsinA indeed has at least one substrate within the NE. These striking findings implicate the presence of torsinA substrates in two different cellular locations and suggest a possible link between nuclear envelope function and dystonia pathogenesis.

Recent research by Goodchild, Kim, and Dauer has established a structural manifestation of the torsinA Δ GAG mutation (Goodchild et al., 2005). In both TOR1A knockout mice ($TOR1A^{-/-}$) and Δ GAG mutation knock-in mice ($TOR1A^{\Delta GAG/\Delta GAG}$), cells of the CNS develop severe NE ultrastructural abnormalities and exhibit a large

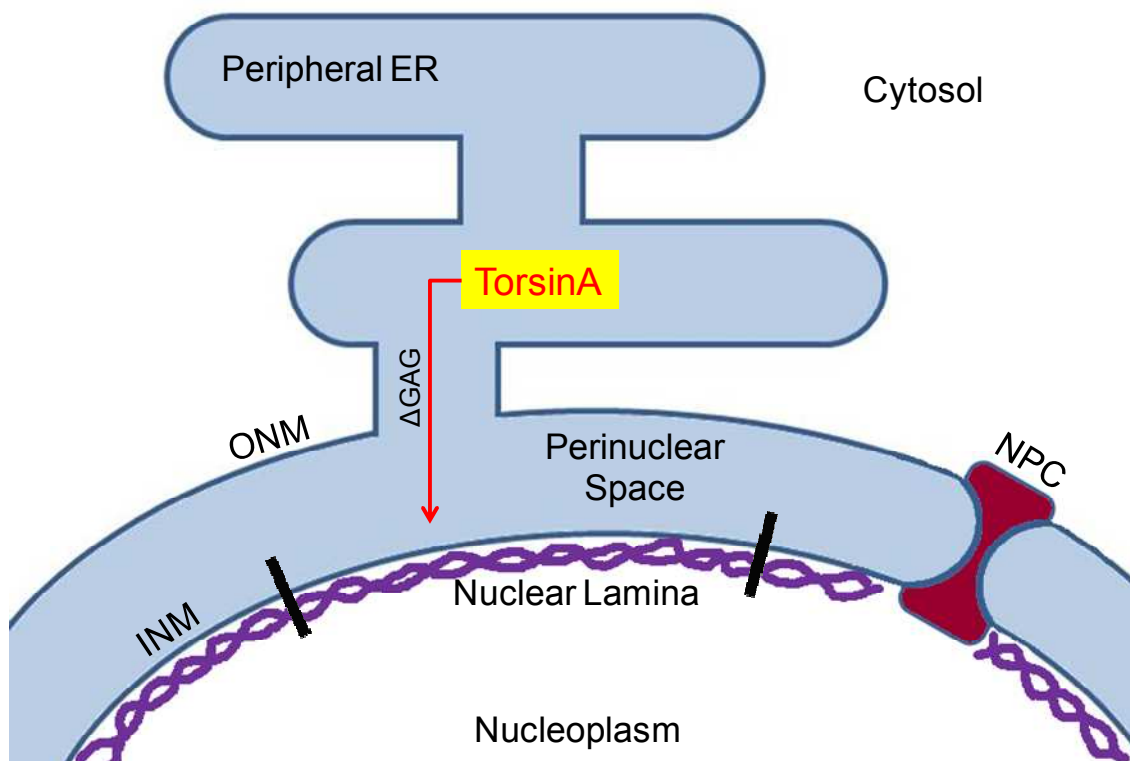


Figure 1.1. Schematic of the NE and peripheral ER. The red arrow indicates the relocation of torsinA in response to the Δ GAG mutation present in DYT1 dystonia.

number of perinuclear vesicles and INM blebs not seen in WT cells. These abnormalities are seen only in cells of the CNS, with large ventral horn neurons being the most severely affected. The lack of abnormalities in non-neuronal cells, even though these cells express torsinA, implicates a neural-specific function of these vesicles that may lead to dystonia. Further, the similar appearance and manifestation between knockout and knock-in mice strongly suggests that the Δ GAG mutation is a loss-of-function allele. Finally, NE ultrastructural abnormalities increase in a rostral-caudal direction that mimics the progression of CNS development and strongly corresponds with the childhood-onset nature of DYT1 dystonia.

1.2.4. Identification of TorsinA Substrates

In the past years, transmembrane proteins of the nuclear lamina and ER have been identified as substrates of torsinA (Goodchild and Dauer, 2005). Overexpression of the lamina protein LAP1 (lamina-associated peptide) causes the recruitment of a large amount of torsinA to the NE with a uniform perinuclear distribution, indicating that it is a torsinA substrate. The LAP family of lamina proteins are integral membrane proteins that have a fundamental role in membrane attachment and assembly of the nuclear lamina (Martin et al., 1995). The luminal domain of LAP1 is highly conserved among eukaryotes, indicating that torsinA interacts with a highly conserved domain of LAP1 (Goodchild and Dauer, 2005). Additionally, the novel transmembrane ER protein LULL1 (luminal domain like LAP1) has been identified as a substrate for torsinA. LULL1 shares a highly conserved luminal domain with LAP1, with significant divergence occurring only in the regions outside of the lumen. The identification of torsinA substrates in two distinct cellular

locations implies that both the NE and ER may be critical in dystonia pathogenesis. Cells from DYT1 patients show a marked reduction in protein secretion rate from the ER that may be caused by the impairment of WT torsinA chaperone processes (Hewett et al., 2007). In addition, the role of the nuclear lamina suggests that DYT1 dystonia pathogenesis may be similar to that of the laminopathies, diseases associated with laminal dysfunction (Burke, 2002).

1.3. Electron Tomography to Study Bleb Genesis

The striking formation of perinuclear vesicles and blebs in ΔE -torsinA cells leads to the fundamental question: What is the origin of these structures? Before their function and implications in dystonia pathogenesis can be understood, the mechanics behind their formation must be analyzed. Since the current understanding is lacking, a detailed structural characterization of these blebs is performed in this study. For this analysis, the technique of electron tomography (ET) is employed. ET utilizes the transmission electron microscope (TEM) to produce three-dimensional reconstructions of sub-cellular organelles with nanometer resolution. These properties of ET make it the ideal choice for a structural characterization of the nanometer-scale structural imperfections seen in ΔE -torsinA cells. The discussion of ET begins with a brief overview of tomography followed by a more in depth background of the techniques used to produce reconstructions.

1.3.1. The Need for Electron Tomography

Conventional TEM micrographs possess a variety of drawbacks that make the determination of three-dimensional structures difficult. TEM micrographs are two-

dimensional projections of a three-dimensional specimen, causing features from different levels to be superimposed and reducing clarity (Frey et al., 2006). With conventional TEM operating at 100 kV, thin sections of about 60-80 nm thickness are typically used. Since most cellular organelles of interest are significantly thicker than this, the true determination of their structure from these thin slices is difficult. In addition, a variety of three-dimensional geometries may produce the same two-dimensional projection through a given cross-sectional plane, potentially making conventional TEM micrographs misleading.

Technological advances in electron microscopy have led to the advent of electron tomography, a method for using the TEM to visualize ultra-structural details of cellular organelles. The recent availability of intermediate high-voltage microscopes operating in the 200-600 kV range has allowed for the use of thicker sections on the order of 300-500 nm. This thickness is large enough to encompass a significant portion of structural detail of sub-cellular organelles. The use of a greater voltage increases the mean-free electron path, which allows the imaging of thick sections before scattering effects lead to a significant degradation of image quality.

ET utilizes the mathematical principles of the Radon transform to fill in a three-dimensional density map of the region of interest (ROI) by using the backprojection of tilt images (Frey et al., 2006). A tilt series, or a series of projection images of the ROI at different angles, is collected by incrementally rotating the TEM specimen holder about a fixed perpendicular axis. The raw data from the tilt series is loaded as an image stack and manipulated so subsequent sections are properly aligned. Following alignment, reconstruction can be performed using a variety of techniques. Following the reconstruction of a three-dimensional volume, data can be segmented and

interpreted to provide detailed structural information. (Perkins et al., 1997). An overview of the process of ET, from tilt series collection to surface rendering, is shown in Figure 1.2.

IMOD, a set of programs developed at the University of Colorado, Boulder, is used to display and process tomographic reconstructions (Kremer et al., 1996). The IMOD program *3dmod* is used to view raw image stacks and completed tomograms as well as generate three-dimensional models. The eTOMO graphical user interface (GUI) is used to filter and align raw image stacks and produce reconstructions. An in depth analysis of the functions performed by the various components of the IMOD package is presented in subsequent sections.

1.3.2. Sources of Error in Electron Tomography

A variety of errors and imperfections may be introduced during the process of tilt series collection, and the minimization of these errors is critical for the production of high resolution reconstructions. X-rays and other flaws in CCD camera images can produce extreme low or high values that ruin the contrast and can also give undesirable artifacts in the reconstruction. Sample warping may occur due to prolonged exposure to the electron beam, generating a morphology that may be different than expected. In addition, a variety of TEM alignment problems can occur, and proper alignment steps are imperative before data collection. Finally, the proper translational and rotational alignment of successive projections of a tilt series is critical. If the tilt series is improperly aligned, reconstruction methods will yield very poor results. The process of fine alignment is discussed in detail in the following section.

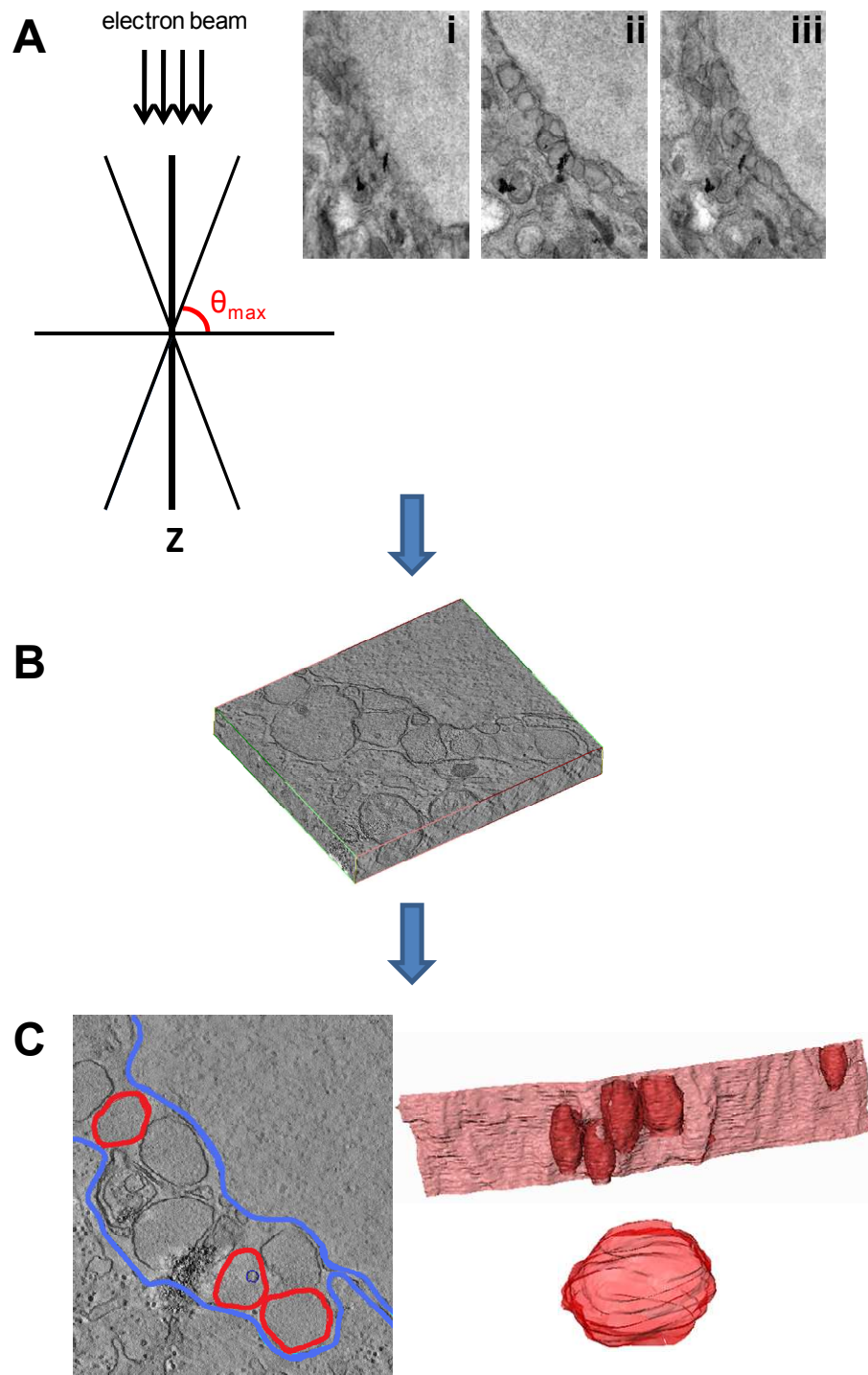


Figure 1.2: Overview of the electron tomography process. (A) Tilt series images are collected at angular intervals around the axis of the electron beam (Z axis). (i) -60° , (ii) 0° , and (iii) $+60^\circ$ projections from an E18 reconstruction. (B) Reconstruction yields a three-dimensional volume of the ROI. (C) Volume segmentation by manual tracing followed by surface rendering generates three-dimensional models that can be manipulated for further information.

1.3.3. TxBR Fine Alignment

A precise alignment of the individual images comprising a tilt series is a critical initial step in the process of obtaining a high quality reconstruction. The goal of alignment is to produce a model of the projections from the actual object to the resulting image. To aid in the process of alignment, gold fiducial markers are applied to both sides of the specimen before imaging and tracked through each tilt projection. Gold fiducial markers are advantageous for tracking because they are easily identifiable due to their large electron scattering density in comparison to the surrounding tissue. In addition, gold particles remain in a fixed position in the specimen throughout tilt series collection. Despite these advantages, misalignment of gold particles is a common source of error in tomographic reconstructions. Misalignment may be due to a number of factors, including poor goniometer eucentricity, sample warping, and sample mass loss (Fan et al., 1995). Optical aberrations and other two-dimensional issues may be eliminated using a variety of known image transforms. However, sample warping, shrinkage, and other disorders due to curvilinear electron trajectories are three-dimensional problems and cannot be eliminated by such transforms (Lawrence et al., 2006).

The TxBR (transform based back projection) software package developed by Lawrence and colleagues in 2006 accounts for the curvilinear trajectories of electrons and helps reduce some of the three-dimensional errors in tomogram alignment (Lawrence et al., 2006). The fine alignment step makes corrections to a user-created fiducial model of gold particles across all tilt angles so that fiducial markers move horizontally in successive tilts. To achieve this, two sets of unknowns must be determined: the three-dimensional positions of the marked gold particles in the object

and their projections onto individual tilt images. The particle trajectory across tilt angles can be considered as the sum of contributions from projective (straight-line) and non-linear trajectories. Projective and non-linear contributions are refined by a bundle adjustment optimization process. The quadratic approximation of each projection, P , along curvilinear electron paths is given by a polynomial with 23 transform coefficients (unknowns) (Lawrence et al., 2006). To solve for this situation in every projection, a large number of fiducial markers are required. The general rule followed is that the total number of two-dimensional fiducial coordinates must be greater than the sum of the number of three-dimensional fiducial coordinates and the number of transform coefficients. In the quadratic case, this inequality is:

$$2NT > 23N + 3T \quad (1.1)$$

where N is the number of tilts collected and T is the number of fiducials tracked. For a quadratic approximation, a general rule of $T \geq 40$ is followed to achieve a reliable reconstruction (Lawrence et al., 2006).

1.3.4. IMOD Reconstruction: The R-Weighted Backprojection

The final volume is reconstructed from a series of two-dimensional projections that intersect along the path of the electron beam (z -axis) and are spaced at equal angular increments about it. Since the projections share a common intersection, the problem of reconstructing a three-dimensional object can be reduced to a series of two-dimensional reconstructions in planes that are perpendicular to this intersection (Gilbert, 1972). The following derivation is shown in two-dimensions for clarity but can easily be extended to three-dimensions. Projections at an angle θ are referred to the

Cartesian coordinates x_θ and y_θ arising from rotation of the standard coordinates x and y by an angle θ :

$$\mathbf{x}_\theta = \mathbf{R}_\theta \mathbf{x} \quad (1.2)$$

The rotation matrix $\mathbf{R}_\theta$ for a counter-clockwise rotation is defined as:

$$\mathbf{R}_\theta = \begin{bmatrix} \cos \theta & -\sin \theta \\ \sin \theta & \cos \theta \end{bmatrix} \quad (1.3)$$

A diagram of the coordinate systems used is shown in Figure 1.3.

Projections are typically taken with values of θ ranging from -60° to $+60^\circ$ in angular increments of 2° , giving 61 total projections (N). The angular increment, $\Delta\theta$, between subsequent projections is given as:

$$\Delta\theta = \frac{4\pi}{3N} \quad (1.4)$$

The total amount of Fourier space covered with such a scheme is $4\pi/3$ radians. Since full coverage requires 2π radians of coverage, this scheme results in some missing

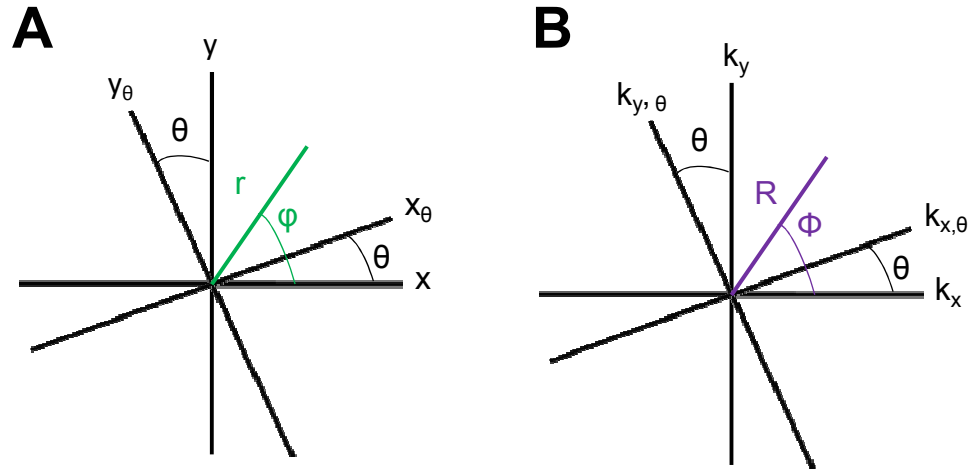


Figure 1.3: Cartesian and polar coordinate systems before and after rotation by an angle θ .
(A) Real and (B) Fourier space.

information in Fourier space. Total coverage would require values of θ ranging from -90° to $+90^\circ$. However, this range is unattainable in ET due to partial or total blockage of the sample by the grid bars at high tilt angles. Additionally, the path of the electron beam through the specimen increases as a function of tilt angle, creating scattering problems at large angles. This missing information in Fourier space resembles a wedge and is referred to as the wedge of missing information, shown graphically in Figure 1.4.

If $\rho(x,y)$ denotes the two-dimensional density distribution of the object being reconstructed, then the two-dimensional Fourier transform of the density distribution, F , is given as:

$$F(k_x, k_y) = \iint \rho(x, y) \exp[-i2\pi(k_x x + k_y y)] dx dy \quad (1.5)$$

The one-dimensional density distribution, $P(x_\theta)$, is obtained by projecting the density of the object along y_θ onto x_θ such that:

$$P(x_\theta) = \int_{-\infty}^{\infty} \rho(x, y) dy_\theta \quad (1.6)$$

In the following analysis, the problem of deducing $\rho(x,y)$ from a series of projections, P , is considered. According to the central slice theorem, the Fourier transform of the projection $P(x_\theta)$ is equal to a slice through the origin of the two-dimensional Fourier transform that is parallel to the rotated projection line. Mathematically, the theorem is stated as:

$$\mathfrak{F}\{P(x_\theta)\} = F(k_x, k_y) \delta(k_{y,\theta}) \quad (1.7)$$

Taking the inverse Fourier transform of (1.7) and summing over all projection angles yields:

$$\sum_{\theta} P(x_{\theta}) = \mathfrak{F}^{-1} \left[\sum_{\theta} F(k_x, k_y) \delta(k_{y,\theta}) \right] \quad (1.8)$$

The summation of the central sections of the Fourier transform on the right-hand side of (1.8) can be rewritten by introducing a sampling function, $S(R, \Phi)$, defined as:

$$\begin{aligned} S(R, \Phi) &= \delta(k_x \cos \Phi + k_y \sin \Phi - R) \\ \Phi &= 4\pi n/3N; n = 1, \dots, N \end{aligned} \quad (1.9)$$

where δ represents the Dirac delta function. The polar angle in Fourier space, Φ , increases by the angular increment of the tilt series. The simple backprojection reconstruction technique utilizes this definition of the sampling function. A problem with the simple backprojection technique arises, however, since the delta function assigns a unit weight to $S(R, \Phi)$ at all locations of Fourier space (Gilbert, 1972). Since the sampling function extends in the radial direction of Fourier space, it must be

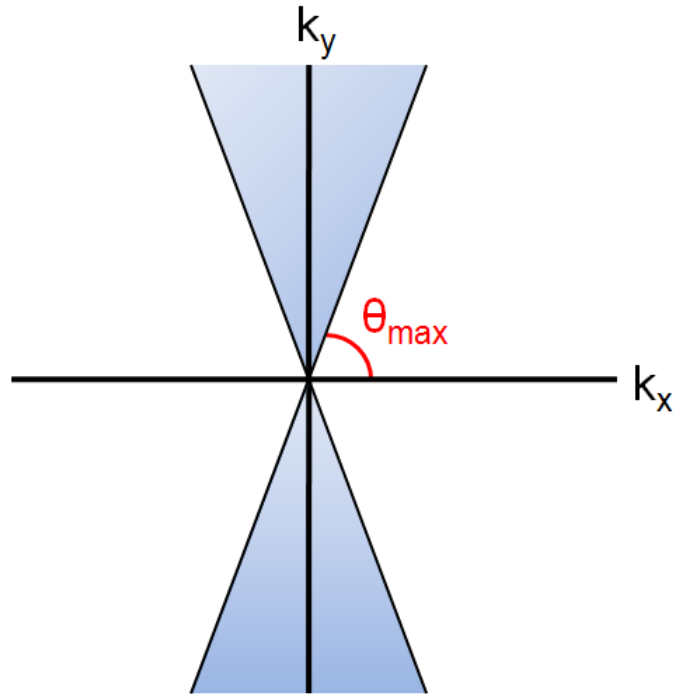


Figure 1.4: The wedge of missing information in Fourier space. The first and last projections occur at $\pm\theta_{\max}$. In a typical tilt series, $\theta_{\max} = 60^\circ$, leaving a total of $2\pi/3$ radians of missing information in Fourier space. The missing wedge is shown in blue.

made to increase with the radial Fourier coordinate R to achieve an accurate reconstruction. The first step towards this goal is to define a set of functions, $G(x_\theta)$ whose Fourier transforms are equal to those of the projections, $P(x_\theta)$, multiplied by the Fourier space radius, R . This occurs when G is defined as follows (Gilbert, 1972):

$$G(x_\theta) = \frac{1}{2\pi^2} \frac{\partial P(x_\theta)}{\partial x_\theta} * \frac{1}{x_\theta} \quad (1.10)$$

Applying the central slice theorem to the definition of G and taking the inverse Fourier transform gives:

$$\sum_{\theta} G(x_\theta) = \mathfrak{F}^{-1} \left[\sum_{\theta} R F(k_x, k_y) \delta(k_{y,\theta}) \right] \quad (1.11)$$

Note the similarity between (1.8) and (1.11). The right-hand side of equation (1.11) is re-written by introducing the sampling function, S :

$$\sum_{\theta} R F(k_x, k_y) \delta(k_{y,\theta}) = F(k_x, k_y) R S(R, \Phi) \quad (1.12)$$

The inverse Fourier transform of (1.12) is found by applying the convolution theorem:

$$\mathfrak{F}^{-1} [F(k_x, k_y) R S(R, \Phi)] = \rho(x, y) * \mathfrak{F}^{-1} [R S(R, \Phi)] \quad (1.13)$$

Combining (1.13) and (1.11) gives:

$$\sum_{\theta} G(x_\theta) = \rho(x, y) * \mathfrak{F}^{-1} [R S(R, \Phi)] \quad (1.14)$$

Thus, the reconstructed density, ρ_{rec} , achieved by the process of R -weighted backprojection is defined as the convolution of the true density with the inverse Fourier transform of the weighted sampling function (Gilbert, 1972):

$$\rho_{\text{rec}}(x, y) = \rho(x, y) * \mathfrak{F}^{-1} [R S(R, \Phi)] \quad (1.15)$$

If the number of projections is infinite, all Fourier terms will be included and properly weighted, and a true reconstruction would be expected. This is not possible due to

real world limitations, but the process of weighting each spatial frequency proportional to its Fourier space radius produces a more faithful reconstruction than the simple backprojection method.

1.4. Objectives

The use of ET allows for the novel analysis of ultrastructural defects in the nuclear envelope seen in dystonia models with a torsinA Δ GAG mutation. Since the torsinA mutation is a loss-of-function allele, cells without torsinA exhibit the same structural manifestations as those with the mutation. Due to the CNS-specific manifestation of dystonia, torsinA knockout (KO) neurons from the cervical spinal cords of E18 rats are the primary cell types studied. Additionally, it is unknown whether the NE defects are due to simple torsinA loss or if they are caused by the change of function of a torsinA substrate. LAP1 is a substrate of torsinA at the nuclear envelope. To compare the loss of LAP1 function with the loss of torsinA function, LAP1 knockout DIV5 neurons are also analyzed. Finally, LAP1 KO human embryonic kidney cells (HEK) are studied to explore the effect of loss of function in other systems of the body and to determine if the same defects found in neurons are present in different cell types. Thus, a total of three cell types are studied: E18 neurons (torsinA KO), DIV5 neurons (LAP1 KO), and HEK cells (LAP1 KO). In this study, the following questions are addressed:

1. Bleb Genesis: Do NE blebs bud from nuclear pores? If so, is a single pore involved or are multiple pores caught up in the process? Genesis from a single pore implies a stereotyped process whereas genesis incorporating multiple pores implies a random process in which pores are caught up.

2. Perinuclear Vesicle Genesis: Do blebs remain attached to the NE via a membranous neck or truly bud off as vesicles?
3. TorsinA and LAP1 Loss: How do NE ultrastructural defects compare between the three cell types? Are bleb and vesicle genesis similar in LAP1 and torsinA KO cells? Is genesis different in neuronal versus non-neuronal cells?

CHAPTER 2: METHODS

2.1. Sample Preparation

Blocks of cervical spinal cord tissue from E18 torsinA KO mice as well as blocks of HEK cells and DIV5 neurons were received from the laboratory of Dr. William Dauer, a collaborator from Columbia University. Sections from each of the blocks were cut at thicknesses of 400-500 nm and applied to clamshell grids by Dr. Guy Perkins. Cut sections were stained 30 minutes in 2% aqueous uranyl acetate followed by 15 minutes in lead salts to prepare for EM viewing. Fiducial markers consisting of colloidal gold particles with diameters of 15 nm and 20 nm were deposited on both sides of each section. Following poststaining and gold application, grids were ready to be imaged.

2.2. TEM Setup

Microscopy was performed using a JEOL 4000EX intermediate-voltage electron microscope. The microscope voltage was increased from a resting value of 300 kV to the operating voltage of 400 kV in increments of 0.1 V/s. The sample rod was removed from the specimen holder of the microscope column and placed on a Plexiglass holder. Grids were loaded into the first and second grid locations so that their grid bars were aligned parallel to the long axis of the rod. Once loaded, the rod was inserted back into the specimen holder of the column. Before turning on the filament current, a delay of fifteen minutes was required for reestablishment of the proper column vacuum. The dark current was increased to the maximum operating value of ~122 pA.

2.3. Region of Interest Selection

A strict set of criteria were established for the selection of a region of nuclear envelope for tilt series collection. First, only nuclei that contained a large number of perinuclear blebs were imaged. Second, both the INM and ONM were required to be clearly visible and defined across the entire region of interest (ROI). Third, only ROI that occur near the middle of each grid box were selected to prevent blockage at high tilt angles. The middle of each grid box was approximated by determining the distance between the grid bars on opposing sides and dividing this distance in half. The center of each grid box was then scanned for intact nuclei. Scanning was performed at a low magnification, typically 5,000x. Once an intact nucleus was found, the magnification was increased to between 20,000 and 30,000x to look for the presence of blebs and integrity of the nuclear membranes. When a nucleus with abundant blebs and intact nuclear membranes was found, the tilt angle was changed to $\pm 60^\circ$ to ensure grid bar blockage did not occur. If there was no grid blockage, the ROI was selected for tilt series acquisition and the tilt angle was reset to 0° .

2.4. TEM Alignment

2.4.1. Gun Tilt, Condenser Stigmation, & Condenser Shift

TEM alignment was performed over the ROI to precondition the area to the electron beam, thus decreasing specimen thinning during tilt series collection. To provide maximum illumination, the tilt of the gun filament was aligned with the optical axis of the condenser lenses. The magnification was set to 5,000x and the electron beam was spread out beyond the capacity of the screen to undersaturate the filament. The electron gun beam deflector coil current was varied until the highest

current density was achieved, signifying maximum illumination. Without proper alignment, the electron beam was elliptical in shape and illuminated the specimen surface unevenly, a phenomenon caused by astigmatism in the condenser lenses. To correct this astigmatism, the current in the condenser lens stigmator coil was varied. Current was varied until the beam became as circular as possible on both sides of crossover and the distortion due to astigmatism was minimized.

Following gun tilt alignment, the electron beam may still be shifted in the x and y directions of the condenser lenses and must be centered with the optical axis. Leaving the magnification at 5,000x, the focus screen was brought out to aid in centering. With the electron beam set at spot size 1, the beam was condensed to a fine point just before crossover. The electron gun beam deflector coil current was varied and the electron beam was translated in the x and y directions until it was centered exactly on a black dot in the center of the focus screen. Following centering, the spot size was changed to 4 and the beam was again condensed to a fine point just before crossover. The condenser lens beam deflector coil current was varied and the electron beam was translated in the x and y directions until it was centered on the focus screen. This process was repeated iteratively, switching back and forth between spot sizes 1 and 4 and correcting the beam until it remained centered following a change in spot size, indicating the minimization of beam shift.

2.4.2. High Voltage Centering

High voltage centering was performed to align the beam parallel to the optical axis at high magnifications. The magnification was increased to 50,000x and a nearby gold particle was brought into focus with the aid of the X or Y wobbler. The

DEFLECTOR: BRIT TILT setting was employed to tilt the electron beam when observing a bright field image. The condenser lens beam deflector coil current was varied while bringing the gold particle in and out of focus. When improperly aligned, the gold particle moved linearly when changing focus. Current was altered until the gold particle remained as stationary as possible when bringing it in and out of focus. Following centering, the gold particle was brought back into proper focus using the X or Y wobbler.

2.4.3. Objective Aperture Alignment & Objective Stigmatism

Proper objective aperture alignment is critical for obtaining high resolution images. The objective aperture was aligned in the microscope's diffraction mode. The electron beam was first condensed to a small point before entering diffraction mode. The focus of the resulting objective aperture diffraction image was focused by varying the diffraction focus knob. The objective aperture controls were used to align the objective aperture with the spot from the diffraction image. Objective astigmatism was corrected by first locating a gold particle at 100,000x magnification and bringing it into focus with the X or Y wobbler. The gold particle was brought into and out of focus by adjusting the coarse alignment and the current applied to the objective lens stigmator coil was adjusted until the gold particle did not smear when its focus was adjusted.

2.5. Eucentricity & Cooking Routines

The goniometer eucentricity was adjusted to ensure the ROI stayed in roughly the same position when changing the tilt angle. To begin adjusting eucentricity, a large dark object was located and centered on the screen at a tilt angle of zero

degrees. The tilt angle was increased to 10° , and the dark object was re-centered on the screen using the stage controls. Following centering, the tilt angle was changed to -10° and the object was re-centered using small incremental changes with the goniometer adjustment knob and stage controls. This process was then repeated at 10° and repeated iteratively between the 10° and -10° tilt angle pairs until the object remained centered on the screen when switching between the two angles of the pair. This process was repeated at tilt angle pairs of $\pm 20, 30, 40, 50$, and 60° to achieve eucentricity throughout all angles used in the tilt series.

Following all alignment and eucentricity routines, the sample was put through a cooking routine to further precondition the sample to the electron beam before beginning tilt series collection. The electron beam was spread out to irradiate the area of the full screen while maintaining the greatest current density possible. The sample was left in this state at tilt angles of 0° , 60° , and -60° for fifteen minutes at each angle.

2.6. Tilt Series Collection

Tilt series were typically collected beginning at a tilt angle of -60° and progressing to $+60^\circ$ in two degree increments. However, a larger tilt angle range was used in circumstances where the grid bars did not obstruct the ROI. Part of the TEM field of view was displayed in real-time using a video rate camera monitor. Prior to tilt series collection, a prominent feature, usually a gold particle was physically marked on the video rate monitor with a sticky note. This feature was tracked when changing between angles and the stage controls were adjusted so the feature closely lined up with the marker on the monitor. This provided a high degree of initial translational alignment of the raw image stack. With four of the E18 tilt series, images were

recorded on film at 20,000x magnification, developed, and digitized using a Nikon CoolScan 9000 scanner at 1800 dpi. For HEK and DIV5 tilt series, images were recorded via CCD camera at 12,000x magnification and saved at each tilt angle into a raw image stack using the SerialEM software suite (University of Colorado, Boulder). Prior to recording at each tilt angle, the image was brought into coarse and fine focus with the assistance of an X/Y image wobbler and the current density was adjusted to fall within the range of 20-30 pA/cm².

2.7. Tomographic Reconstruction

2.7.1. Pre-Processing

Raw image stacks for each collected tilt series had an original X, Y, Z size of 4096 x 4096 x Z, where Z is the number of projection angles. Raw stacks were binned by a factor of two using the stack editor *newstack* (Imod). Binning decreases the nm/pixel resolution by a factor of two but also significantly decreases processing time. The resulting binned stacks had an X, Y, Z size of 2048 x 2048 x Z. The periphery of binned image stacks possessed a great deal of large CCD flaws. Binned image stacks were cropped to a final X, Y, Z size of 1996 x 1996 x Z to cut out these flaws. Cropping was performed with *newstack* and did not result in significant data loss since the areas of interest were centered in the XY plane.

Binned and cropped raw stacks were loaded into eTOMO with a pixel size of 2.42 nm/pixel (corresponding to a magnification of 12,000x), average gold fiducial diameter of 17 nm, and image rotation of 90 degrees. Undesirable CCD and x-ray artifacts were eliminated using *ccderaser*, an eTOMO routine that produces an IMOD model file marking flawed pixels in the stack. This routine searches for and corrects

regions of the raw image stack that possess large intensity spikes. An example of the function of this routine is shown in Figure 2.1. Each projection was divided into patches and the mean and standard deviation (SD) of the pixels in each patch were computed. When a pixel was found to deviate from the patch mean significantly, all neighboring pixels were scanned to find the peak of intensity. Next, the mean and SD of a subset of pixels within a given radius of the peak were calculated. If the peak varied from the subset mean by greater than 10 SDs, the pixel corresponding to the peak was marked for correction in the IMOD model file. Pixels within each patch were also scanned for extreme deviation from their neighbors. The difference between each pixel and the mean of the surrounding eight neighbors was calculated, and the mean and SD of the differences were computed for the entire patch. If the difference

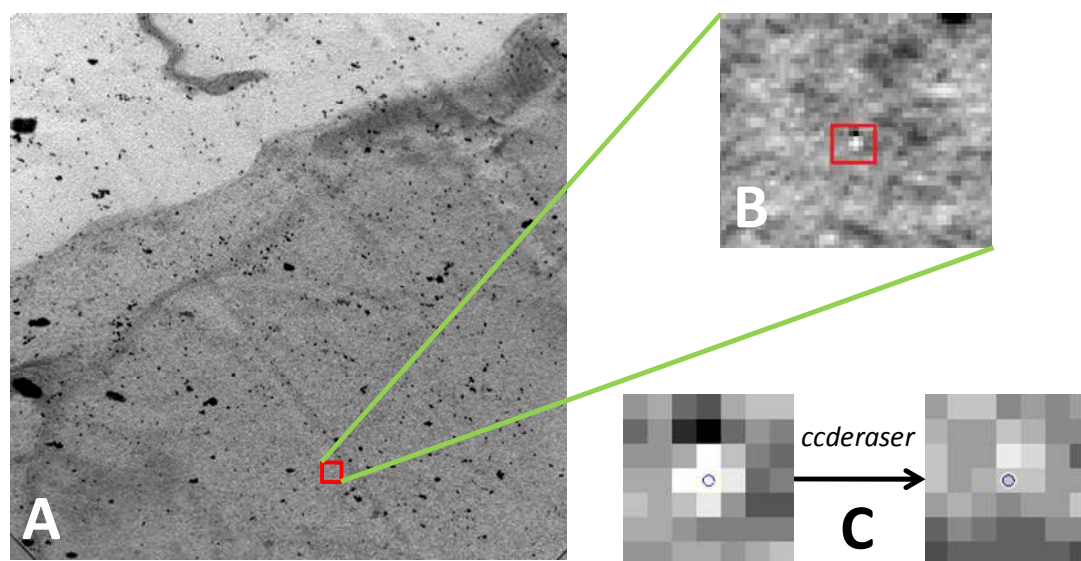


Figure 2.1: IMOD's *ccderaser* routine locates and removes large intensity spikes due to CCD flaws. A single slice of a HEK raw image stack (A) is magnified by a factor of five to show a CCD imperfection (B). Zooming in by a factor of 20 shows individual pixels and demonstrates the intensity spike before and after smoothing with *ccderaser* (C).

of a pixel deviated from the patch mean by more than 8 SDs (the difference criterion) this pixel was also marked in the IMOD model file for replacement. After scanning of all projections was complete, all errant pixels marked in the model file were replaced by fitting a polynomial to adjacent pixels and interpolating a new value from this polynomial.

2.7.2. Coarse Alignment

Coarse alignment of the tilt series consisted of two steps: user-adjusted alignment with the Midas GUI of eTOMO followed by computer-based alignment with cross-correlation. Using Midas, pairs of successive images were overlaid and given different colors to easily differentiate them. The first image (reference) was held fixed while the second image was rotated and translated. Rotations and translations were performed until gold particles from both images were aligned along the horizontal and spaced equally on both sides of the axis. The process of alignment using Midas is demonstrated in Figure 2.2. Following alignment with Midas, the IMOD module *tiltxcorr* was run to perform alignment based on cross-correlation. The cross-correlation function (CCF), Φ , is given by (Frank, 1980):

$$\phi_{lm} = \sum_j \sum_k i_1(j,k) i_2(j+l,k+m) \quad (2.1)$$

In this equation, i_1 and i_2 are the optical densities of successive images in the tilt series. In 1982, Guckenberger found that the best cross-correlational alignment was achieved when the image with the larger tilt angle was stretched in the direction perpendicular to the tilt axis by the ratio of the cosines of the two tilt angles (Guckenberger, 1982). This stretch was applied prior to calculation of the CCF. Images were filtered using a high-pass Gaussian filter with $\sigma = 0.05$ and a cutoff

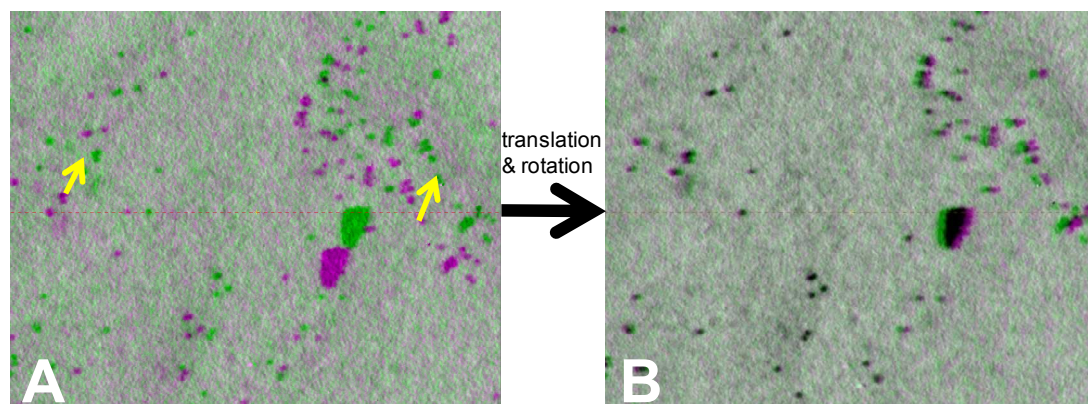


Figure 2.2: Midas provides user controlled coarse alignment before proceeding to cross-correlational alignment. The current image (green) is aligned with an overlay of the previous image (purple) so that gold particles lie on the same horizontal line in successive images. The yellow arrows in (A) show the combination of translation and rotation that is needed achieve the proper alignment of (B).

radius of 0.25 cycles/pixel. Additionally, to reduce the effects of noise, images were filtered using a low-pass Gaussian filter with $\sigma = 0.03$. Successive images of a tilt series show a peak in the CCF array at a distinct location. The vector pointing to this peak from the CCF origin is the desired shift. The second image was shifted by a vector of equal magnitude and opposite direction to attain coarse cross-correlational alignment.

2.7.3. Fiducial Model Generation

A fiducial seed model was created at the zero degree tilt slice of the coarse aligned image stack. The zero tilt projection was chosen for seeding because the gold particles were most easily visible and distinct from the background at this angle. The fiducial seed model consisted of at least 40 contours, with one point per contour manually placed in the perceived center of a distinct gold particle. This resulted in the seeding of at least 40 different gold particles. The seed model consisted of a roughly

50/50 distribution of gold particles on the top and bottom surfaces of the specimen. The surface location of each gold particle was determined by observing the behavior of the particle when scanning upwards through the projections of the stack. Images on different surfaces moved in opposite directions when moving through the projections, making surface differentiation a simple task. Following placement of initial points, the seed model was refined by zooming in by a factor of about eight and placing each point precisely in the middle of the corresponding gold particle.

Following model generation, the seed model was filled in so the gold particle corresponding to a given contour was marked with a point on every projection. This was either done manually or using the IMOD routine *beadtrack*. This routine works from one projection to the next and deduces the position for the gold particle across all projections by using cross-correlation. The size of the search box used to look for the expected position of the gold particle was 48 x 48 pixels. Following automatic tracking with *beadtrack*, the model file was checked to ensure that each fiducial was marked on each projection. When a gap existed, the gap was filled in by manually placing an additional point on the gold particle. Points that were placed far away from the actual position due to tracking errors were manually moved to the center of the gold particle. The end result was a fiducial model with T fiducials tracked across N projections. For a standard tilt series from -60° to $+60^\circ$, $T = 40$ and $N = 61$.

2.7.4. Fine Alignment

Fine alignment was performed with the TxBR software package. Alignment was performed using a quadratic alignment transform without pitch correction. TxBR was run iteratively until a suitable alignment was achieved. The first TxBR alignment

run was performed with a bundle adjust perturbation value of 10 and maximum standard deviation of 2. The suggested fine alignment modifications were saved to an IMOD log file and loaded. The Bead Fixer feature of IMOD was used to cycle through each suggested modification. Bead Fixer displays a vector pointing from the original position of the marker to the location of the suggested improvement. The suggested improvement was implemented if it placed the marker closer to the true position of the gold particle's center and ignored if it did not. Following the first TxBR alignment run, subsequent routines were performed using a bundle adjust perturbation value of 2. This setting was used to detect smaller alignment errors. Following every run, the IMOD log file was loaded and beads were fixed as described above. This procedure was repeated iteratively until the quadratic mean square residual was less than 0.5.

2.7.5. Tomogram Positioning

Before proceeding to reconstruction, the tomogram was shifted and rotated to make it as flat as possible. This was done by analyzing three regions of a sample tomogram generated in IMOD. Small sample tomograms from the top, middle, and bottom of the image stack were generated in eTOMO. A boundary model consisting of two straight lines, one across the top and one across the bottom of the tomogram thickness was created for each sample tomogram. An example boundary model from an E18 reconstruction is shown in Figure 2.3. Following boundary model creation, the eTOMO script *tomopitch* was run. Tomopitch analyzes the boundary model and makes recommendations for how thick to make the slices (tomogram thickness), how much to shift the tilt axis to produce centered slices (Z shift), how much to change the tilt angles to create a flat section (angle offset), and how much X-axis tilt is required to

make the section flat in the orthogonal direction (X-axis tilt). These parameters were applied to the tomogram and the *tiltalign* script was run to generate a final tomogram alignment that incorporated these alterations.

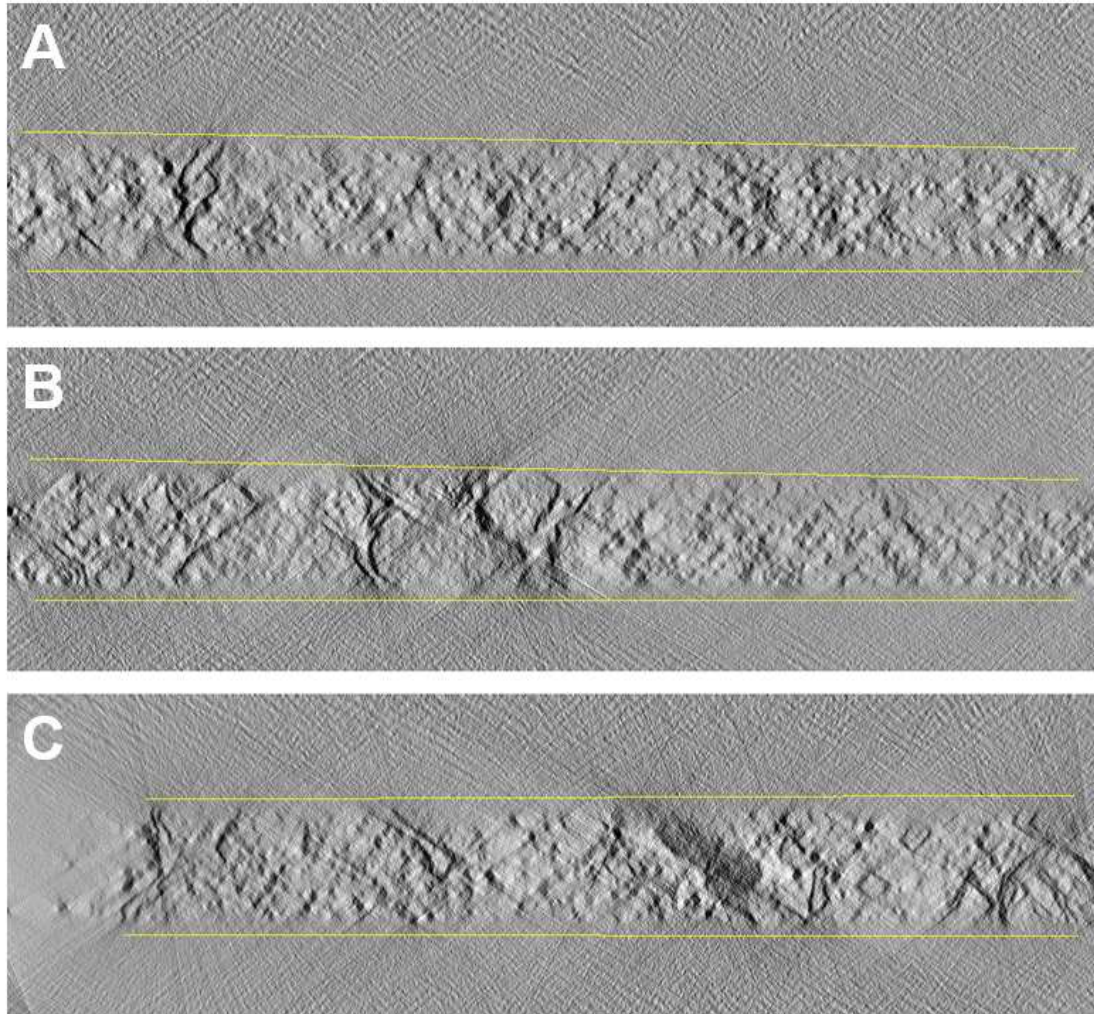


Figure 2.3: Sample tomograms used by *tomopitch* for tomogram positioning. Sample area are bounded on both sides by a yellow line, forming a boundary model. (A) Top sample tomogram, (B) middle sample tomogram, and (C) bottom sample tomogram of an E18 torsinA KO nuclear envelope.

2.7.6. Tomogram Generation

The final aligned stack was initially filtered in Fourier space using a two-dimensional Gaussian filter with cutoff of 0.35 cycles/pixel and fall off of 0.05 sigma. The result was a low pass filtering of the aligned stack to help reduce high frequency noise in both dimensions. Another stage of filtering was applied during the R-weighted backprojection process to filter out additional high frequencies in the reconstruction. Without filtering, each line of input data is filtered to weigh each spatial frequency proportional to its Fourier space radius. This relation is a simple line through the origin. To cut out high frequencies, a Gaussian filter with cutoff of 0.21 cycles/pixel and fall off of 0.03 sigma was applied. The cutoff frequency specifies the frequency at which the linear relationship changes to a falling Gaussian curve. The filtering and R-weighted backprojection were applied and the final tomogram was generated by the eTOMO *tilt.com* script.

2.8. Single Particle Analysis

2.8.1. Subvolume Selection & Boxing

The preliminary step in single particle analysis is to select subvolumes from final reconstructions that contain necks or pores. Final processed tomographic reconstructions of the nuclear envelope were visualized in three-dimensions using IMOD. Each reconstruction was scanned through every z-slice for the presence of necks or pores. The supposed center of each structure was approximated visually. The positive (+) direction of the subvolume is defined as the direction of increasing z, while the negative (-) direction is that of decreasing z. An illustration of this convention is shown for a representative nuclear pore in Figure 2.4. A strict set of

criteria were adopted for the selection of necks and pores. Pores are defined as a temporary break in both the inner and outer nuclear membranes for which the following criteria are satisfied:

1. The connection of the inner and outer nuclear membranes on both sides of the break is apparent and consistent through the entire thickness of the pore.
2. The inner and outer membranes must be re-formed continuously in both the (+) and (-) directions following the transient break.

Necks require a more stringent set of criteria due to their greater variability in size and shape:

1. The bleb budding from the INM must be visible from top to bottom throughout the reconstruction and not cut off at the beginning or end.
2. The bleb must form a clearly defined membranous neck budding from the INM.
3. The INM must be re-formed continuously in both the (+) and (-) directions following the neck.

Once all the pores and necks from a single reconstruction were identified, individual subvolumes were cut out of the larger volume of the reconstruction by the process of boxing. Subvolumes were boxed from their reconstructions using the UNIX command-line routine *boxstartend* (Imod). Subvolumes were centered on the neck or pore of interest and had a volume of $N \times N \times N$ voxels. The value of N varied depending on the position of the neck or pore within the reconstruction volume. For example, if a pore or neck sat deep within the z-dimension, a large box size was not

possible. EMAN requires subvolumes with X and Y dimensions as powers of two, so N was made to be a power of two whenever possible (typically 64 or 128).

2.8.2. Subvolume Alignment

Due to the natural curvature of the nuclear envelope and random orientation of pores and blebs in space, the axis of the pore or neck was randomly oriented in the XY plane following boxing. Before averaging, it was necessary to ensure the subvolumes were aligned identically. Un-aligned subvolumes showed a “birds-eye view” of the pore or neck in the XY plane, parallel to the nucleocytoplasmic axis. In the first step of alignment, subvolumes were rotated in EMAN so the pore was positioned exactly on the horizontal (Tang et al., 2006). An initial guess for the angle of rotation, θ , was made and applied to the subvolume. Following rotation, the subvolume was viewed in IMOD. If the pore laid exactly on the horizontal following

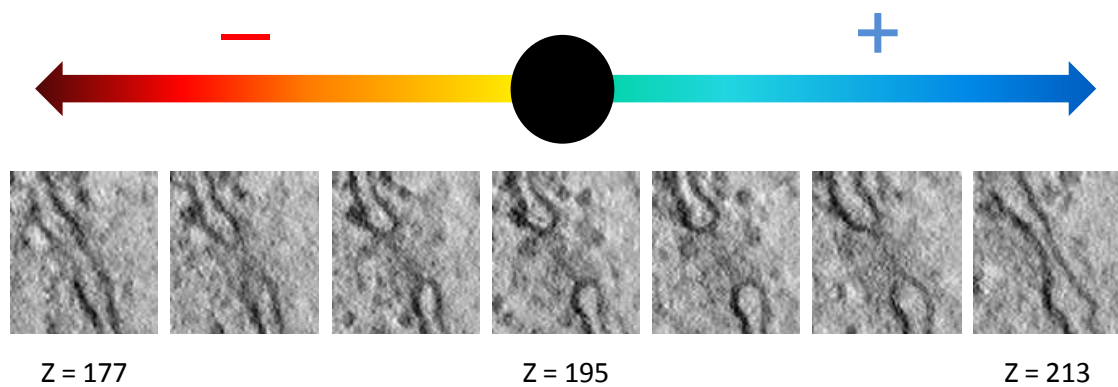


Figure 2.4: Sequential steps through the boxed subvolume of a nuclear pore. The middle slice represents the slice with the suspected largest diameter, as determined through inspection. As the slices progress towards the (+) and (-) ends of the subvolume, the spacing decreases and the pore closes, re-forming continuous membrane on both ends.

rotation, no further alterations were made. If further rotation was needed, θ was increased or decreased until the pore was properly aligned.

Following rotational alignment in the XY plane, subvolumes were further rotated and cropped to go from a birds-eye view of the pore to a head on view of the pore, perpendicular to the nucleocytoplasmic axis. Subvolumes were loaded into Analyze (Biomedical Imaging Resource, Mayo Clinic) and visualized in three-dimensions. Each subvolume was trimmed in the x, y, and z directions to show only the pore and cut out as much extraneous information as possible. Following trimming, the subvolume was rotated 90° about the x-axis to obtain a view through the pore. For better understanding, an illustration of the process of subvolume alignment is shown in Figure 2.5.

2.8.3. Mathematical Averaging & Symmetrization

Following subvolume alignment, separate averages for pores and necks of each cell type were created with EMAN. Only subvolumes showing clear structural details were included in their respective averages; subvolumes with poor structure were likely to adversely affect the average and were discarded. Averaging was performed with the *avg3d* script of EMAN. Following averaging, eight-fold symmetry was imposed on the final pore and neck averages using the EMAN command *proc3d* to obtain further structural information.

2.9. Diameter Measurements

Pore and neck diameter measurements for each cell type were made using NIH ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda,

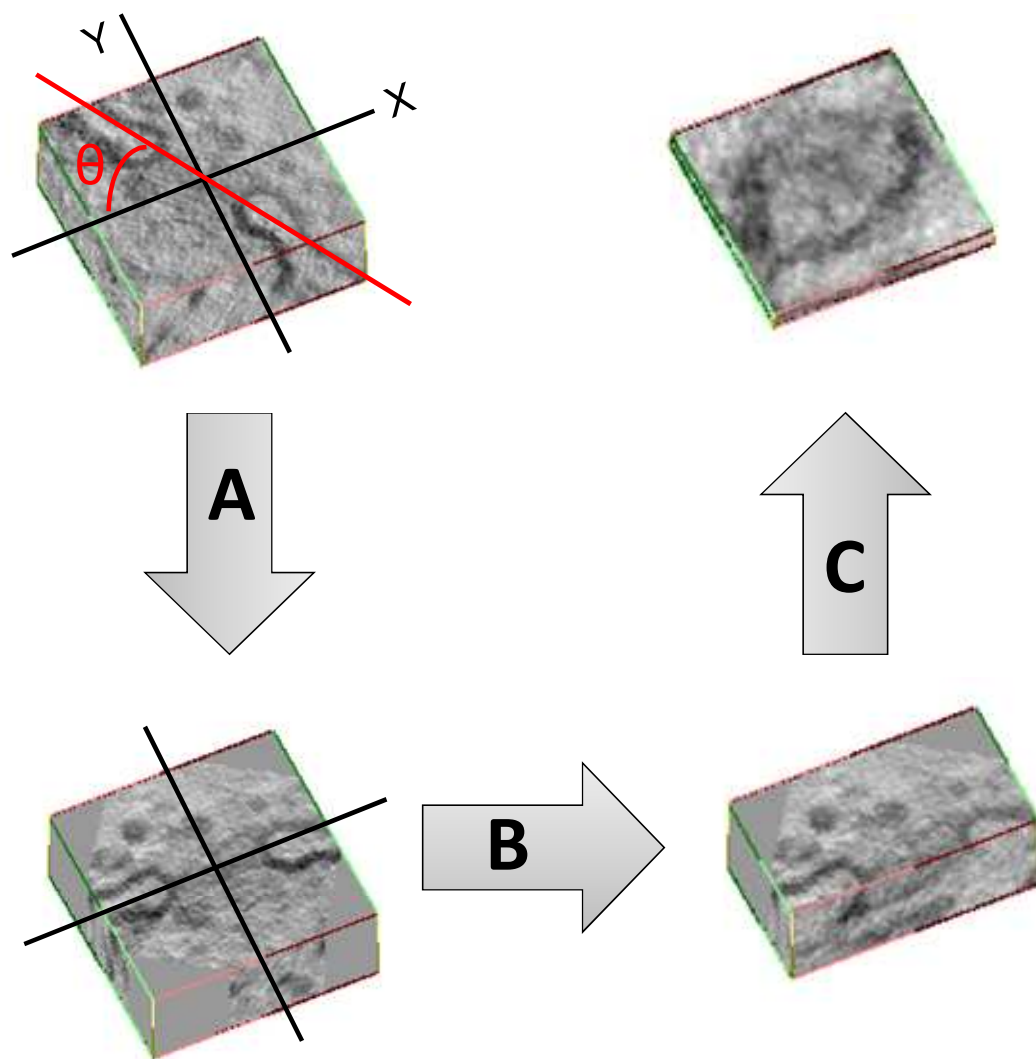


Figure 2.5: Step by step illustration of subvolume alignment using EMAN with an E18 torsinA KO nuclear pore. (A) The birds-eye view of the pore opening was aligned along the x-axis by applying a rotation by an angle θ . (B) Once aligned along the x-axis, volume in the y-direction was cut away using Analyze. This revealed the head on structure of the pore in slices perpendicular to the XY plane. (C) The subvolume was rotated by 90° about the x-axis and cropped to reveal the head on structure of the pore.

Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2004). Measurements were taken for each subvolume in the XY plane. Since measurements were made in the XY plane, the location of the exact center of the pore or neck (i.e. the slice with the largest diameter) was difficult. Pixel length measurements of the pore or neck opening were made on the presumed center slice and the next three slices in both the (+) and (-) directions of the subvolume, for a total of seven slices. Since measurement of such small structures has the potential to produce highly varied results, the length was measured five times per slice and a slice average was produced. This ensured a greater degree of repeatability of results. Once this procedure had been repeated for each of the seven slices from a given subvolume, the maximum length measurement of the seven slices was chosen as the representative maximum diameter measurement for the whole subvolume. To convert from pixels to SI units, the measurements from ImageJ were multiplied by the nanometer-per-pixel resolution of the reconstruction.

2.10. Volume Segmentation

Volume segmentation was performed using the manual tracing tool Xvoxtrace (Perkins et al., 1997). Manual tracing of blebs and vesicles was performed in the XY plane on every fourth slice. The surface-rendering graphics of Synu were used to view the three-dimensional segmentations and measure their volume and surface area (Hessler et al., 1996). Since blebs bud from the INM and are not truly bounded by a membrane about 100% of their periphery, the traces of blebs were closed in the slices where they bud from the INM to allow for volume estimation. The INM was manually traced in every fourth slice to allow for INM surface area calculation to

determine pore and neck densities. For representation in movies, the INM and ONM were traced on every slice and blebs and necks were traced on every second slice. Movies of the segmented volumes were made using Amira (Mercury/TGS, San Diego, CA). Movie features included an overlay of the segmentation through the reconstructed volume, a fully segmented NE with transparent ONM to illustrate bleb and vesicle structure, and transparent blebs to illustrate inner structural details. The overlay of the segmentation through the reconstructed volume creates the perception that the segmentation is being traced in real-time while scanning through the slices of the reconstruction.

An adaptation of the text and figures in this chapter will be submitted for publication with the following co-authors: Guy Perkins, Mark Ellisman, William Dauer, Guido Gaietta, and Tom Deerinck. The thesis author was the primary contributor to the work of this chapter.

CHAPTER 3: RESULTS

3.1. Tilt Series Collection & Reconstruction

A total of five E18, thirteen HEK, and three DIV5 tilt series were recorded. Four of the five E18 tilt series were recorded on film; all other tilt series were recorded using CCD camera. The reconstructed nuclear pores in HEK cells were often low in number or of poor quality and not useful for averaging. Thus, six of the thirteen HEK tilt series were recorded on regions of nuclear envelope that did not contain blebs to maximize the volume available for nuclear pores. Additionally, thinner sections of 300 nm enabled better visualization and were used for the majority of HEK tilt series. Information regarding image size, number of tilts and tracks, magnification, resolution, and binning for each tilt series collected is shown in Table 3.1.

The reconstructed volumes from tilt series recorded on film showed greater detail than those recorded using CCD camera. The time required for collection of a single tilt series using CCD camera was slightly over one hour while the time required when using film was much greater due to the need to exchange film boxes. The number of CCD artifacts detected with *ccderaser* was highly variable. The average was roughly 50 per tilt series with the majority of artifacts falling within one SD of the patch mean. Coarse alignment with Midas and cross-correlation produced stacks that were well-aligned, showing gold particles moving in a roughly horizontal direction across each section. TxBR fine alignment produced a mean square quadratic residual less than 0.5 within about 3-4 iterations. The average square quadratic residual showed initial spikes at the highest and lowest tilt angles, generally at angles greater than $+50^\circ$ and less than -50° . These spikes persisted through roughly three

fine alignment iterations after which they disappeared. All fiducial marker tracks were roughly horizontal following fine alignment. Figure 3.1 uses a HEK tilt series as an example to demonstrate fiducial seeding. Graphs showing residual data from a representative HEK tilt series are shown in Figure 3.2. Representative slices from reconstructions of all three cell types are shown in Figures 3.3-3.6 with markers to indicate the presence of blebs and necks. Low magnification images of the nucleus taken at a magnification of 5,000x are included. Low magnification micrographs of E18 torsinA KO nuclei are shown in Figures 3.7 and 3.8

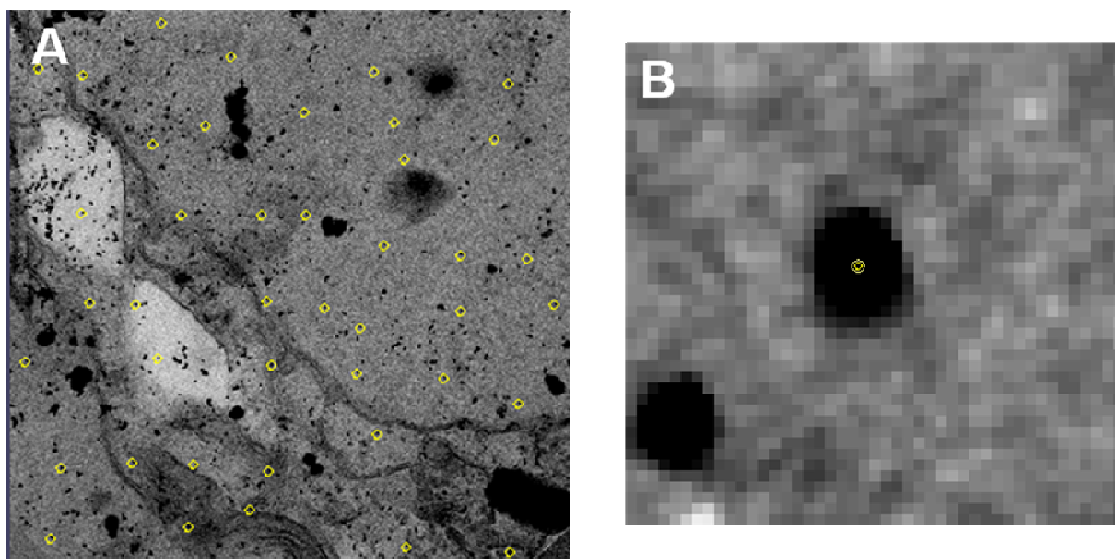


Figure 3.1: Seeding of fiducial markers on representative HEK tilt series. (A) Center slice of a tracked fiducial model demonstrating markers well distributed throughout the XY plane. (B) A single gold particle from the center slice magnified by a factor of eight to demonstrate marker placement in its exact center.

Table 3.1: Information from each tilt series recorded. Binned and unbinned dimensions are reported in pixels in the X and Y directions.

Record Type	ROI	Magnification	TEM Voltage [kV]	Sample Thickness [nm]	Unbinned Resolution [nm/pix]	Unbinned Dimensions (X,Y)	Binning Factor	Binned Resolution [nm/pix]	Binned Dimensions (X,Y)	Tilts	Tracks
E18-1	Film	Blebs & NE	400	500	0.71	4033, 6010	2	1.41	2017, 3006	59	39
E18-2	Film	Blebs & NE	400	500	0.71	4033, 6010	2	1.41	2017, 3006	61	42
E18-3	Film	Blebs & NE	400	500	0.71	4033, 6010	2	1.41	2017, 3006	61	40
E18-4	Film	Blebs & NE	400	500	0.71	4033, 6010	2	1.41	2017, 3006	61	40
E18-5	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	61	40
HEK-1	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	61	40
HEK-2	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	66	45
HEK-3	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	61	105
HEK-4	CCD	Blebs & NE	400	300	1.21	4096, 4096	2	2.42	1996, 1996	61	50
HEK-5	CCD	Blebs & NE	400	300	1.21	4096, 4096	2	2.42	1996, 1996	68	42
HEK-6	CCD	Blebs & NE	400	300	1.21	4096, 4096	2	2.42	1996, 1996	66	43
HEK-7	CCD	Blebs & NE	400	300	1.21	4096, 4096	2	2.42	1996, 1996	66	36
HEK-8	CCD	NE Only	400	300	1.21	4096, 4096	2	2.42	1996, 1996	67	40
HEK-9	CCD	NE Only	400	300	1.21	4096, 4096	2	2.42	1996, 1996	66	42
HEK-10	CCD	NE Only	400	300	1.21	4096, 4096	2	2.42	1996, 1996	67	40
HEK-11	CCD	NE Only	400	300	1.21	4096, 4096	2	2.42	1996, 1996	61	97
HEK-12	CCD	NE Only	400	300	1.21	4096, 4096	2	2.42	1996, 1996	61	40
HEK-13	CCD	NE Only	400	300	1.21	4096, 4096	2	2.42	1996, 1996	61	40
DIV5-1	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	61	35
DIV5-2	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	61	45
DIV5-3	CCD	Blebs & NE	400	500	1.21	4096, 4096	2	2.42	1996, 1996	61	47

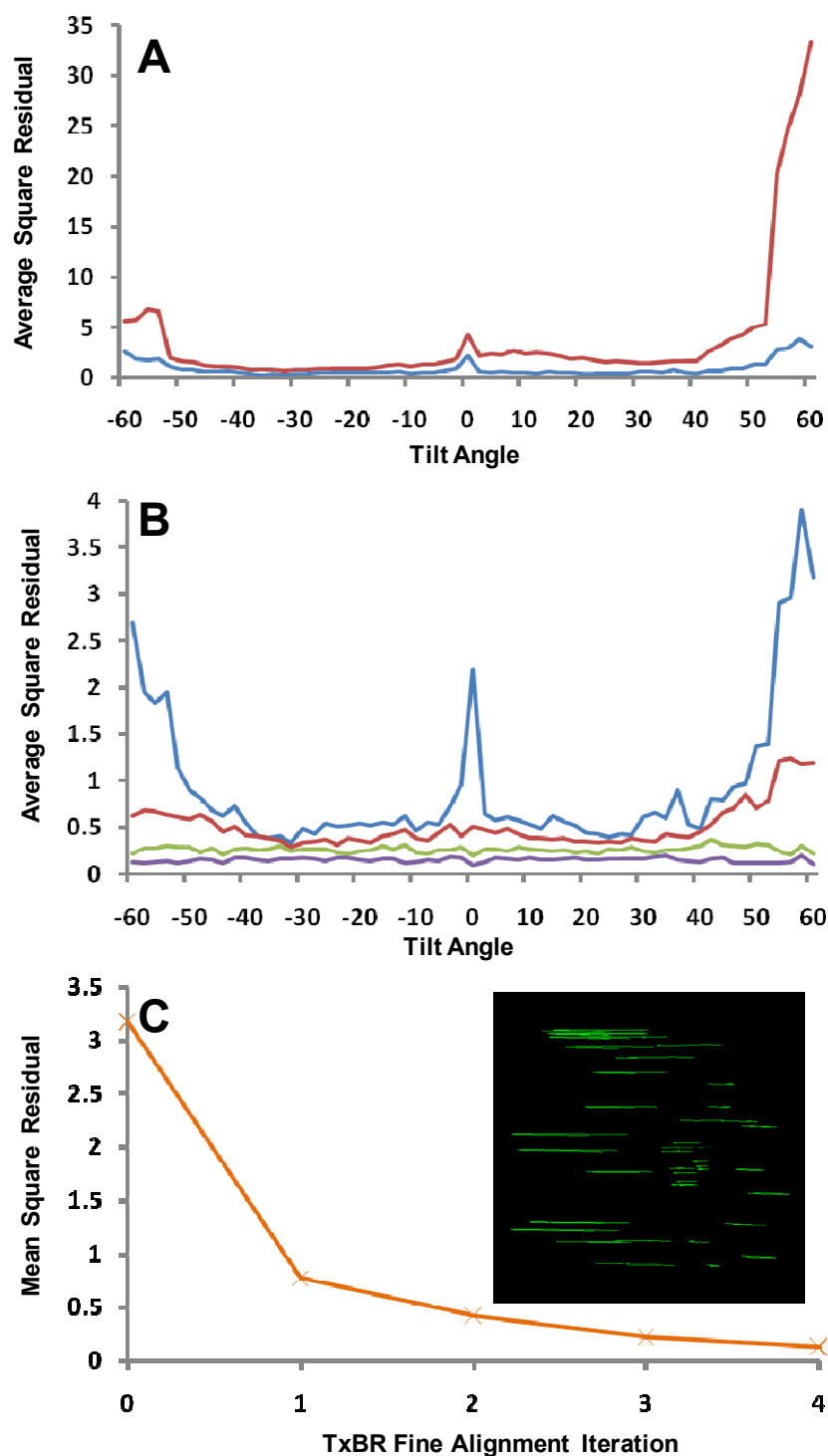


Figure 3.2: TxBR average square quadratic residual versus tilt angle for a HEK reconstruction. (A) Initial residual following tracking (red) and following one TxBR fine alignment iteration (blue). (B) Residual following one (blue), two (red), three (green), and four (purple) TxBR fine alignment iterations. (C) Mean square quadratic residual versus TxBR iteration. Inset: IMOD model file showing horizontal alignment of tracks following alignment.



Figure 3.3: A single slice from a tomogram of an E18 torsinA KO nuclear envelope. Bleb necks are marked with an arrow and nuclear pores are marked with a star. The nucleus is marked with a capital N for clarity.

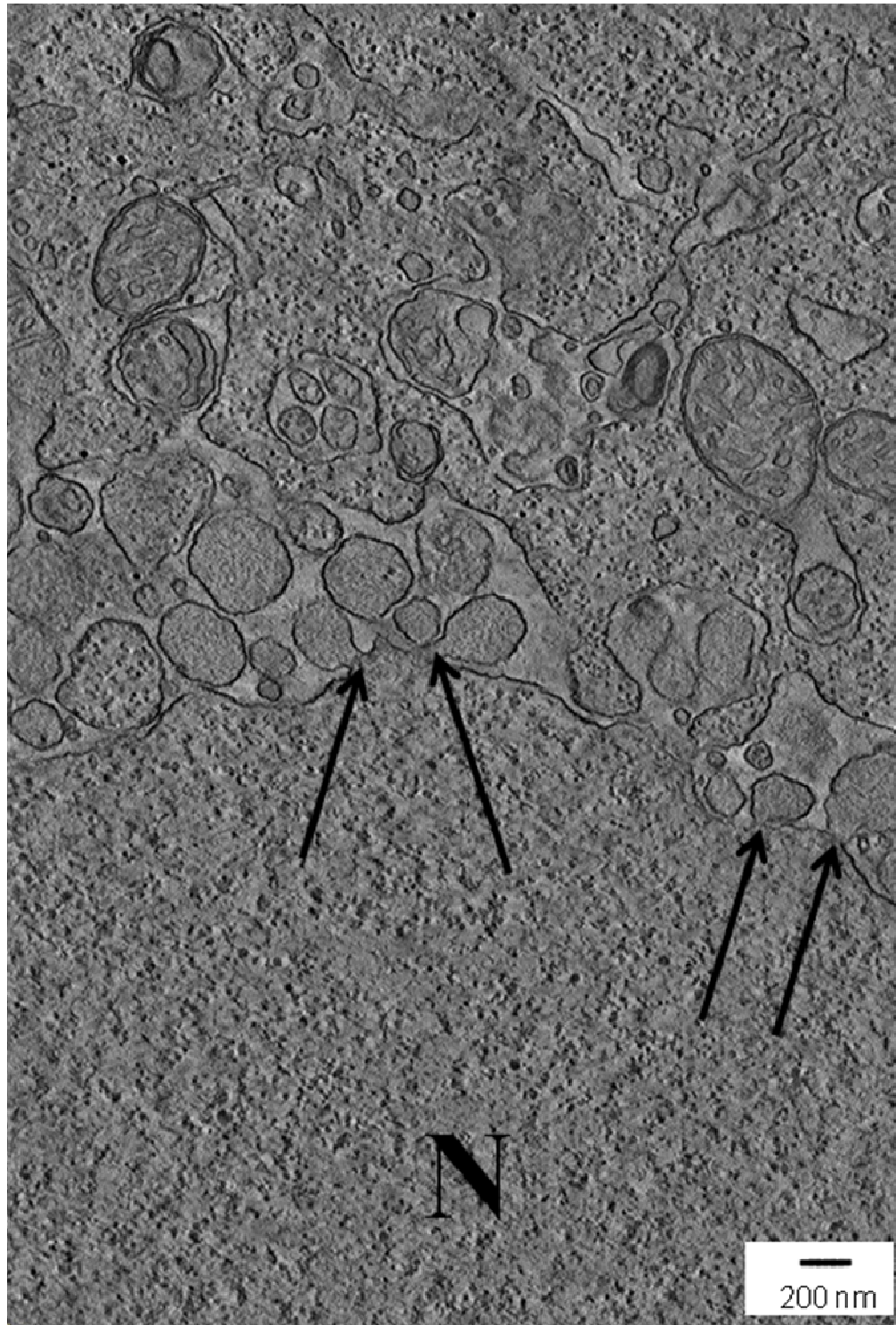


Figure 3.4: A single slice from a tomogram of an E18 torsinA KO nuclear envelope. Bleb necks are marked with arrows.

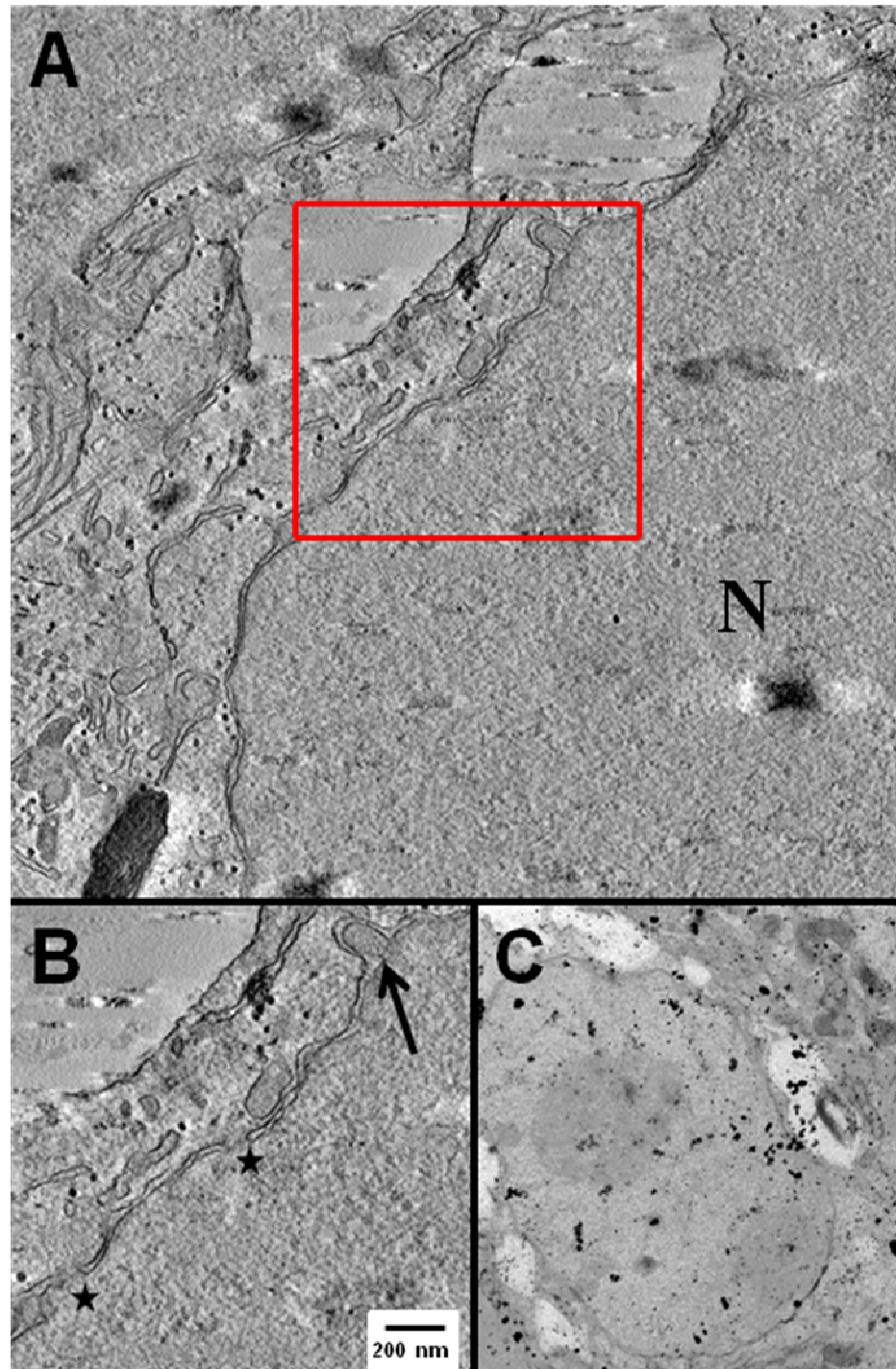


Figure 3.5: Reconstruction of a HEK cell nuclear envelope. (A) Full tomogram at 12,000x magnification. (B) Zoomed in section of the tomogram highlighted by the red box. Nuclear pores are marked with stars and bleb necks are marked with an arrow. (C) Low magnification micrograph of the entire nucleus at 5,000x magnification.

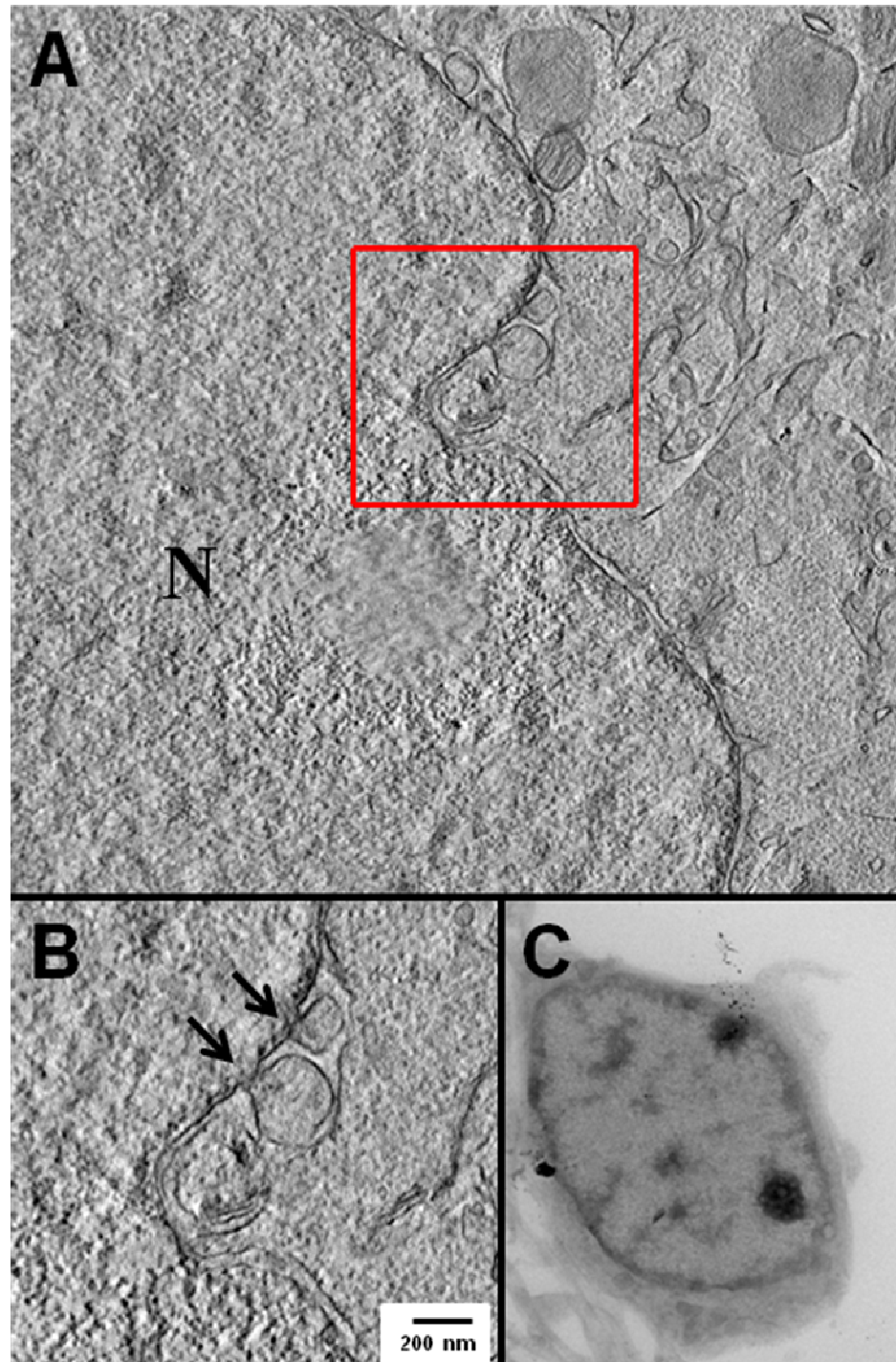


Figure 3.6: Reconstruction of a DIV5 cell nuclear envelope. (A) Full tomogram at 12,000x magnification. (B) Zoomed in section of the tomogram highlighted by the red box. Bleb necks are marked with an arrow. (C) Low magnification micrograph of the entire nucleus at 5,000x magnification.

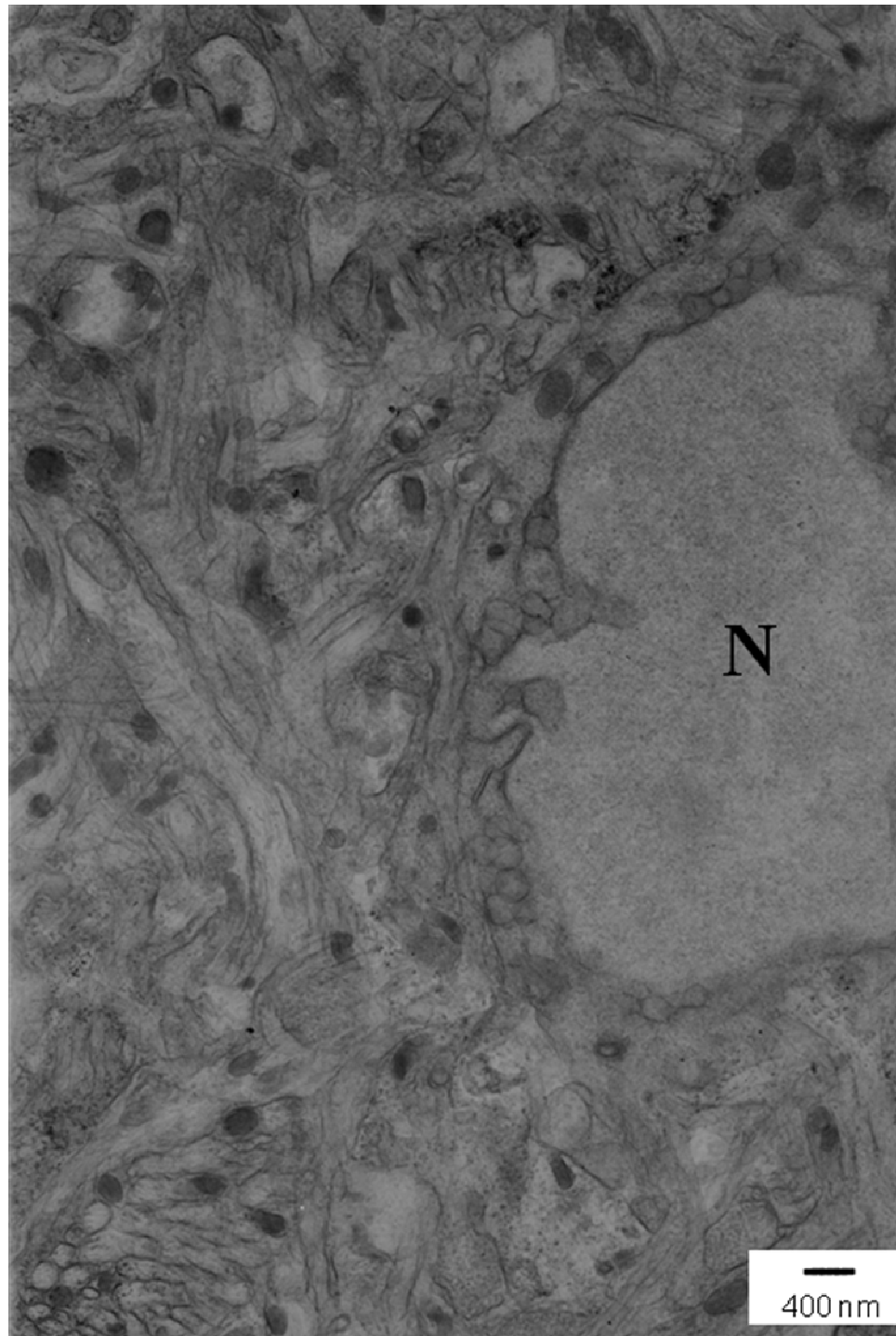


Figure 3.7: Micrograph of an E18 torsinA KO nucleus at 5,000x magnification illustrating blebs and vesicles around its periphery.

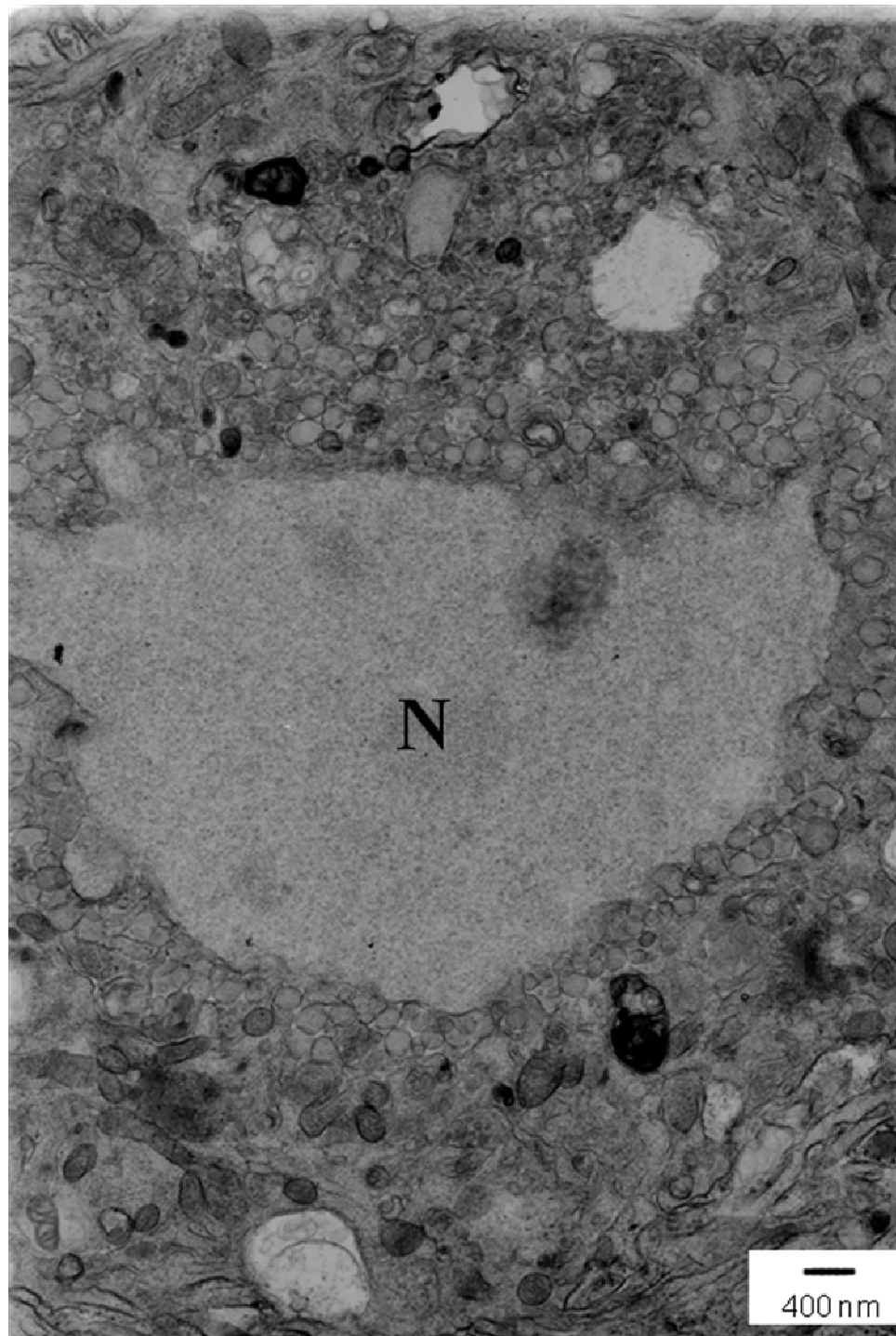


Figure 3.8: Micrograph of an E18 torsinA KO nucleus at 5,000x magnification illustrating blebs and vesicles around its periphery.

3.2. Volume Segmentation, Surface Rendering, & Movie Generation

The INM, blebs, and vesicles of every reconstruction were segmented and surface rendered to provide structural information. A single E18 reconstruction was traced on every XY plane to provide a complete, high quality view of the entire nuclear envelope. The INM, ONM, and select blebs and vesicles were segmented and surface rendered. A movie was generated to show the segmentation of the INM overlaid throughout the reconstruction volume and various angles of the segmented nuclear envelope. An additional movie was generated to show the ultrastructure of a small segment of NE featuring a single bleb. Additionally, movies were created showing sequential slices through the reconstructed volume for every reconstruction produced. Figures of representative renderings are shown in Figures 3.9-3.11.

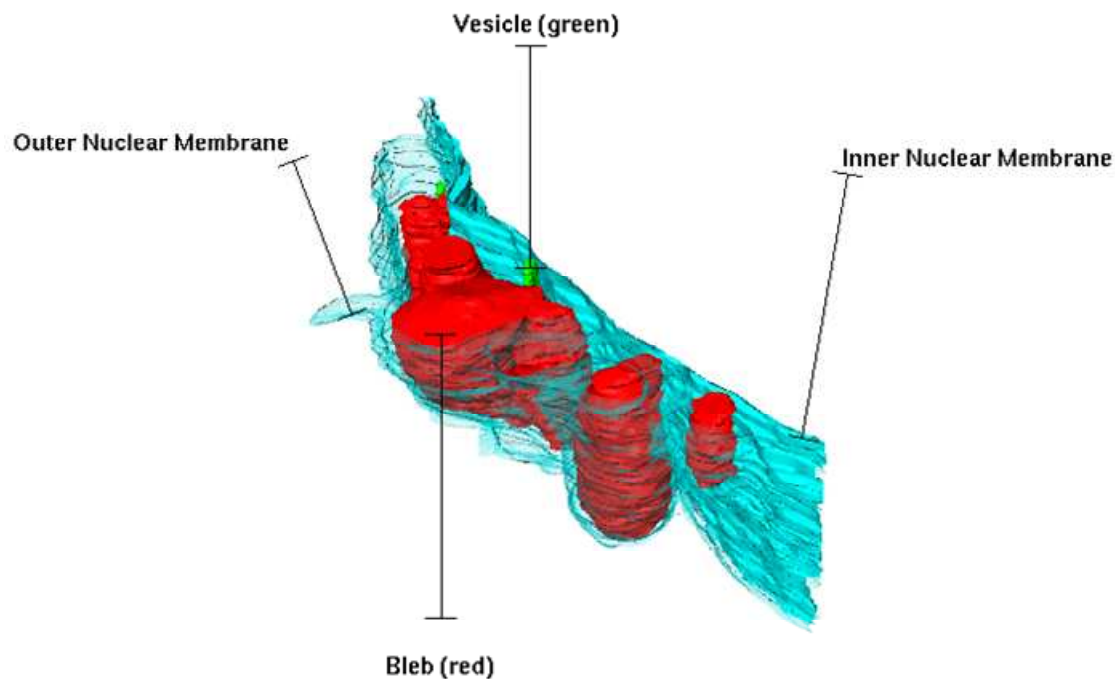


Figure 3.9: Full segmentation of a section of an E18 torsinA KO nuclear envelope. The ONM is translucent to provide a view of the internal structures of the perinuclear space.

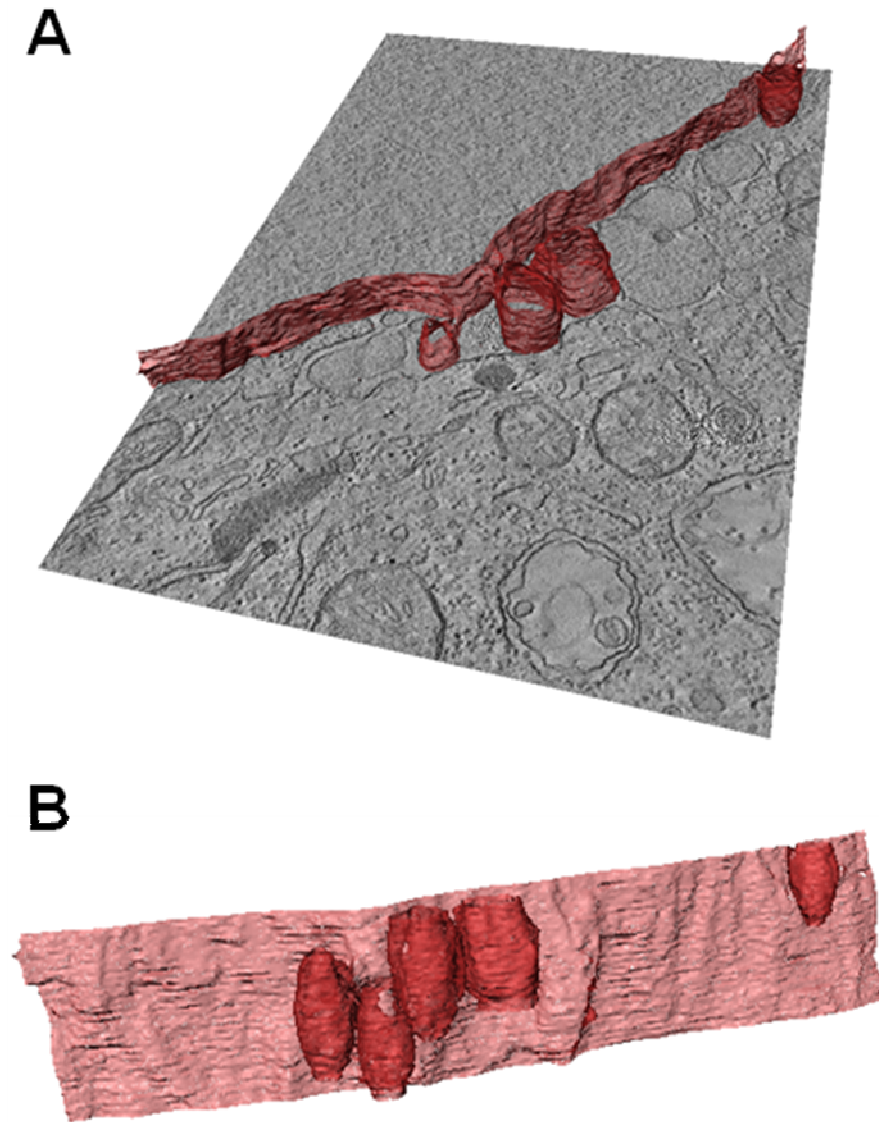


Figure 3.10: INM segmentations from an E18 torsinA KO reconstruction. (A) INM segmentation overlaid on a slice of the reconstruction. (B) Segmentation of the full INM showing a subset of blebs.

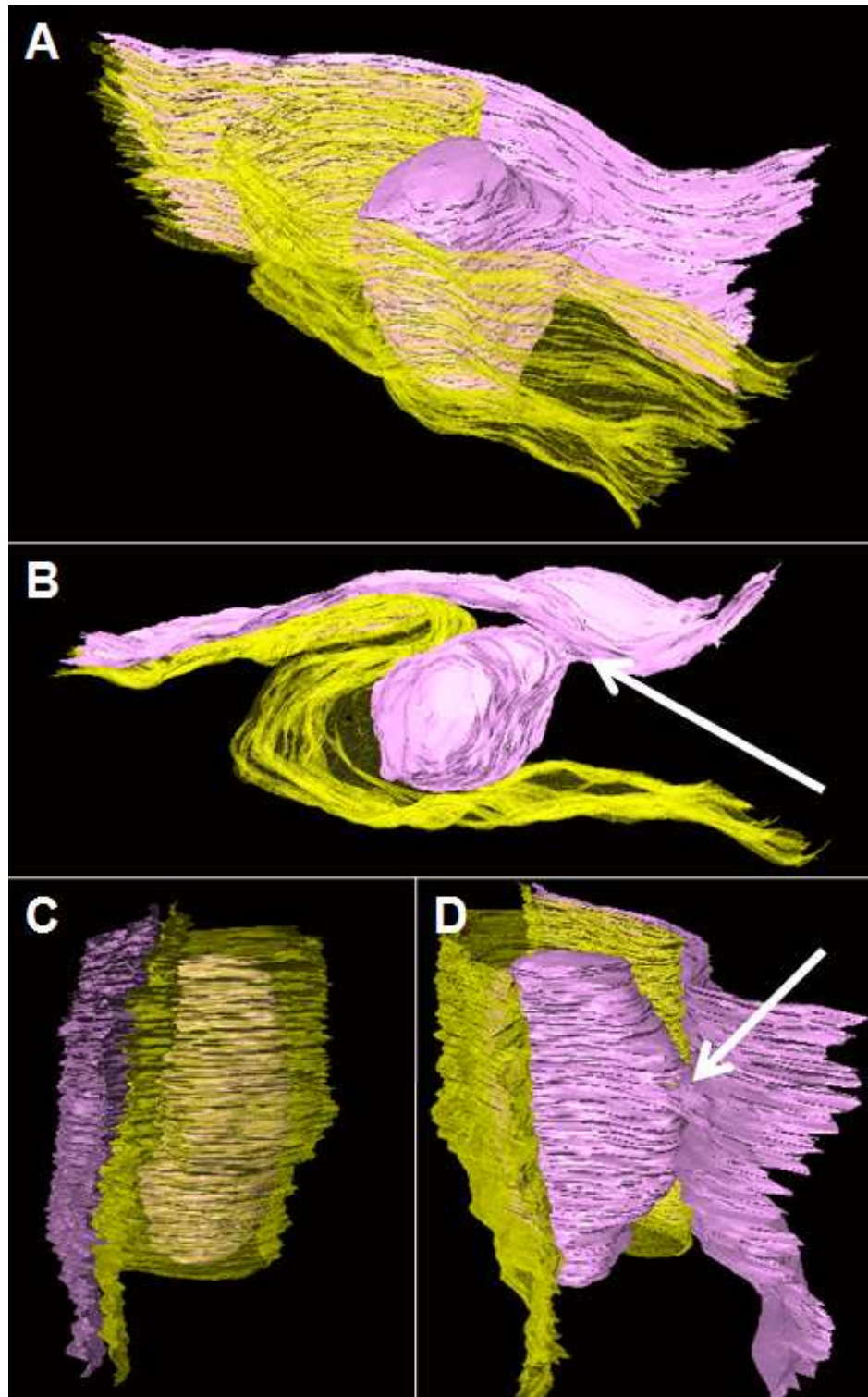


Figure 3.11: Surface renderings of a single bleb and a small segment of NE with a translucent ONM demonstrate neck structure. The INM is displayed in pink and the ONM is displayed in yellow. (A) Front view, (B) top view, (C) and (D) side views. Necks are marked with an arrow.

3.3. *Bleb & Vesicle Morphology*

Vesicles and blebs displayed three distinct morphological classifications: roughly spherical (globular), long and thin (rod-shaped), and flattened (disk-shaped). The rod-shaped morphology was seen only in E18 torsinA KO blebs and vesicles while globular and disk-shaped morphologies were seen across all three cell types. In all three cell types, the globular morphology was dominant. The rough distribution of morphologies per cell type is shown in Table 3.2 and representative surface renderings of blebs and vesicles from reconstructions of each cell type are shown in Figure 3.12. A number of blebs and vesicles could not be segmented due to their interruption by the first or last slice of the reconstruction. These structures were not included in the data of Table 3.2 since their morphologies could not be accurately ascertained without surface rendering.

3.4. *Volume and Surface Area of Blebs & Vesicles*

The volume and surface area of blebs and vesicles were highly variable due to their diverse morphologies. Additionally, volumes varied significantly even within the same morphological category. Because of this variety, the calculation of an average bleb or vesicle volume or surface area is meaningless. To derive a meaningful relation from this information, the volume and surface area of blebs and

Table 3.2: Distribution of bleb and vesicle morphologies across different cell types.

	Globular	Disk-Shaped	Rod-Shaped
E18	62%	16%	22%
HEK	61%	39%	0%
DIV5	65%	35%	0%

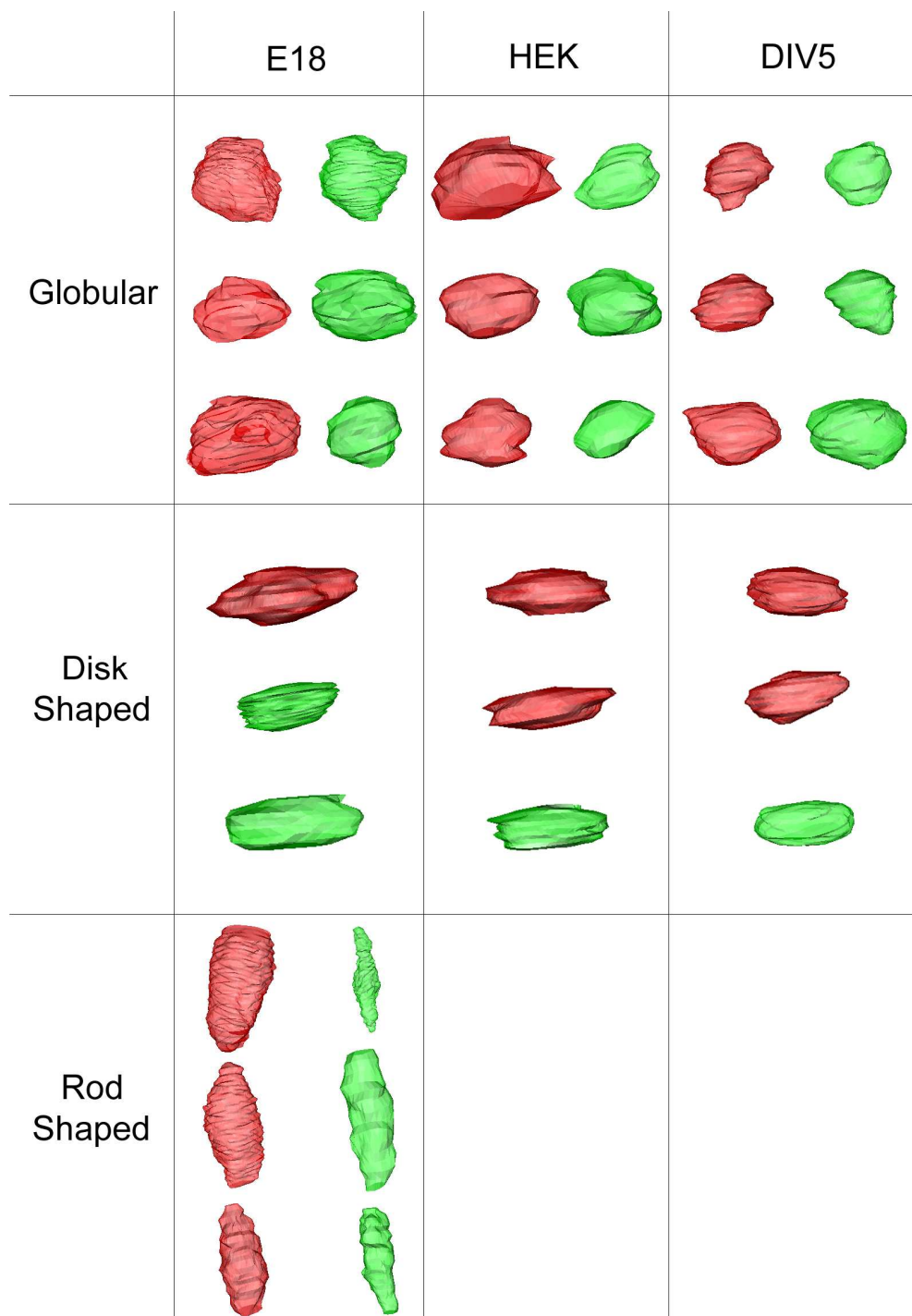


Figure 3.12: Varied morphology of blebs and vesicles from different cell types. Blebs and vesicles are shown in red and green, respectively. Roughly 22% of all E18 torsinA KO blebs and vesicles exhibited a rod-shaped morphology, while HEK and DIV5 blebs and vesicles were devoid of the rod-shaped morphology.

vesicles were compared. The relationship between surface area and volume of both blebs and vesicles of all cell types shows a striking linearity as demonstrated by linear regression analysis. Scatter plots of surface area versus volume data of each cell type are shown in Figure 3.13. Another striking finding is that the surface area to volume (S-V) ratio, defined as the slope of the regression line, is roughly the same between blebs and vesicles of the same cell type, indicating structural similarity. Further, the S-V ratio is roughly the same across all cell types, indicating a stereotyped formation process independent of cell type. Data pertaining to the linear fit of each relationship is shown in Table 3.3.

3.5. Neck & Pore Density

The number of necks and pores present per μm^2 of INM surface area in reconstructions of different cell types is given in Table 3.4. The data is highly variable even amongst the same cell type and no correlation between cell types or between neck and pore density of the same cell type can be drawn. A major cause for this variability was that each reconstruction focused on only a small area of the nuclear envelope rather than its entirety. A region with a high bleb density was often chosen while regions with little to no bleb density were neglected. A reconstruction of the entire nuclear envelope would be required to derive any meaningful relation between neck and pore density, and this would be incredibly time consuming.

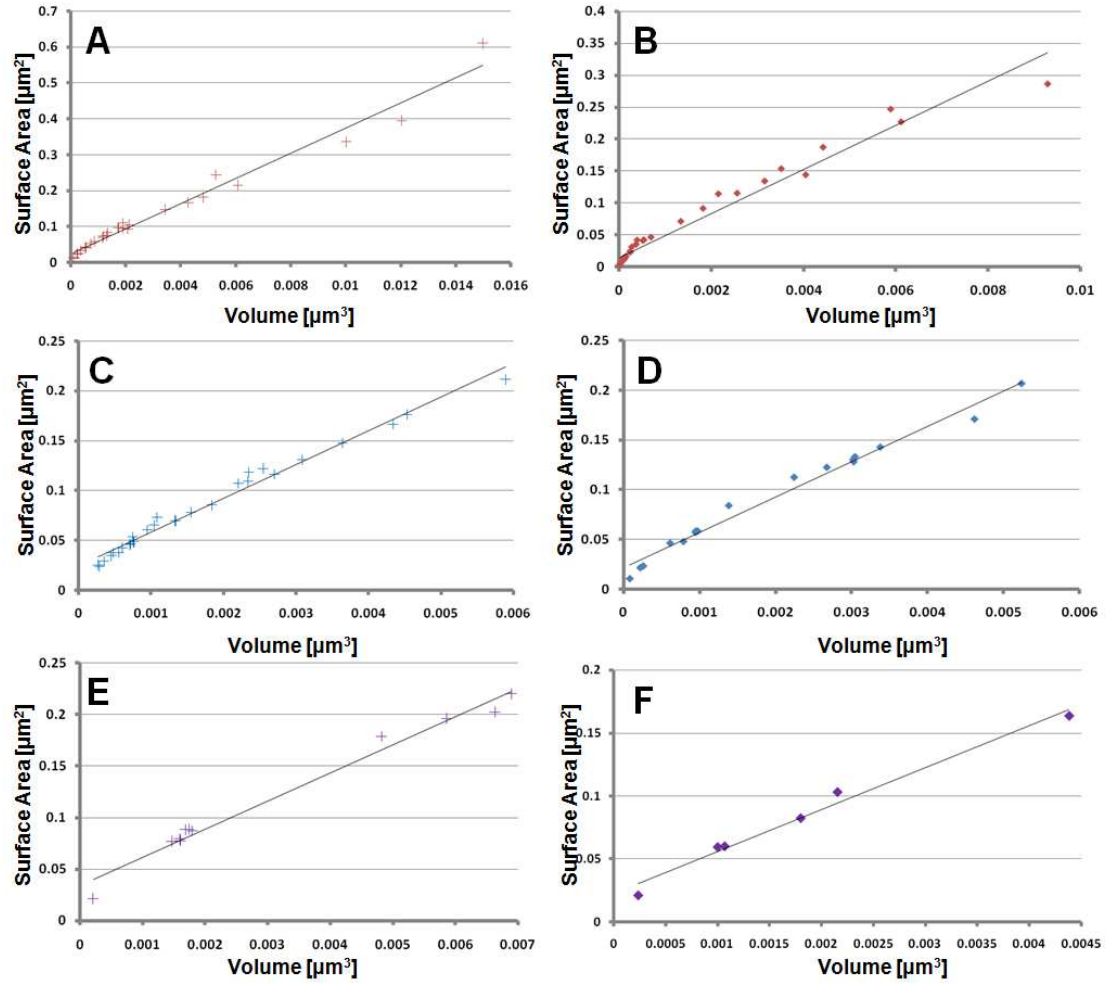


Figure 3.13: Surface area to volume relationships of blebs and vesicles of different cell types. Regression lines are plotted for each relationship. (A) E18 blebs, (B) E18 vesicles, (C) HEK blebs, (D) HEK vesicles, (E) DIV5 blebs, (F) DIV5 vesicles.

Table 3.3: Linear regression statistics of blebs and vesicles for all cell types.

	Slope (SV Ratio)	Y-Intercept	R-squared
E18 Blebs	35.082	0.0241	0.9763
E18 Vesicles	34.617	0.0143	0.9676
HEK Blebs	33.901	0.0243	0.9835
HEK Vesicles	35.624	0.0215	0.9824
DIV5 Blebs	27.253	0.0344	0.9815
DIV5 Vesicles	33.397	0.0223	0.9826

Table 3.4: Neck and pore density for each cell type studied. Density is reported as the number of necks or pores per μm^2 of INM.

E18		HEK		DIV5	
Neck Density [$\#/\mu\text{m}^2$]	Pore Density [$\#/\mu\text{m}^2$]	Neck Density [$\#/\mu\text{m}^2$]	Pore Density [$\#/\mu\text{m}^2$]	Neck Density [$\#/\mu\text{m}^2$]	Pore Density [$\#/\mu\text{m}^2$]
1.16	0.58	1.09	1.09	1.36	3.18
0.71	1.42	0.48	0	0.76	0.76
10.99	4.99	1.25	0.31	0.50	0
2.19	0.36	0	2.18		
		2.13	1.07		
		1.60	0.46		
		5.76	0		

3.6. Neck & Pore Diameters

A bar graph comparing the neck and pore diameters of all three cell types is shown in Figure 3.14. E18 necks and pores have diameters of 68.51 ± 2.22 nm and 67.81 ± 2.80 nm, respectively. HEK necks and pores have diameters of 82.82 ± 4.21 nm and 83.88 ± 4.92 nm, respectively, DIV5 necks and pores have diameters of 91.12 ± 7.46 nm and 95.11 ± 3.89 nm respectively. For all cell types, neck and pore diameters are significantly similar ($P < 0.05$).

3.7. Neck & Pore Averages

Representative slices from three-dimensional symmetrized averages of pores and necks from each cell type are shown in Figure 3.15. In addition, Figure 3.15 depicts a few selected representative subvolumes from both necks and pores illustrating views parallel and perpendicular to the nucleocytoplasmic axis.

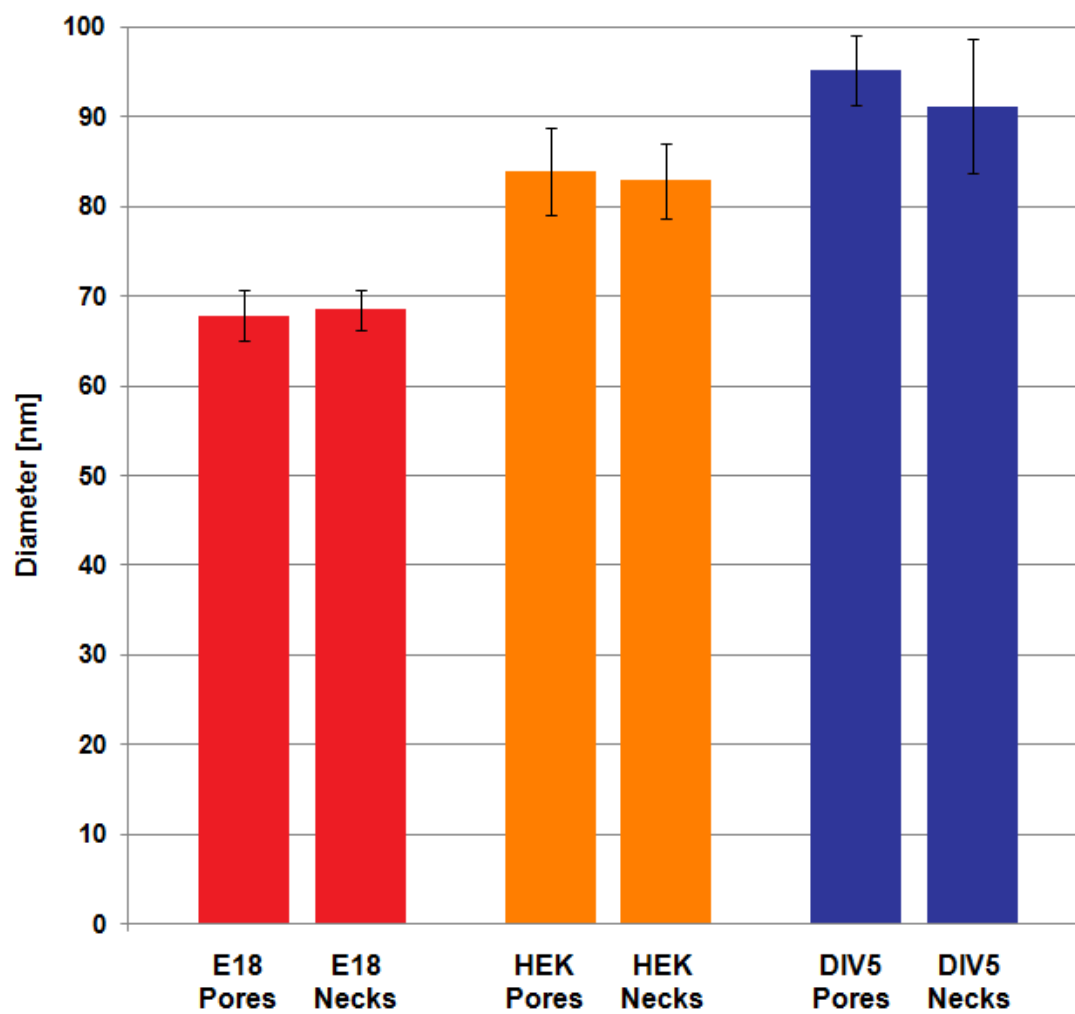


Figure 3.14: Comparison between pore and neck diameters of different cell types. Bars represent the mean value of the diameter and error bars represent the standard deviation.

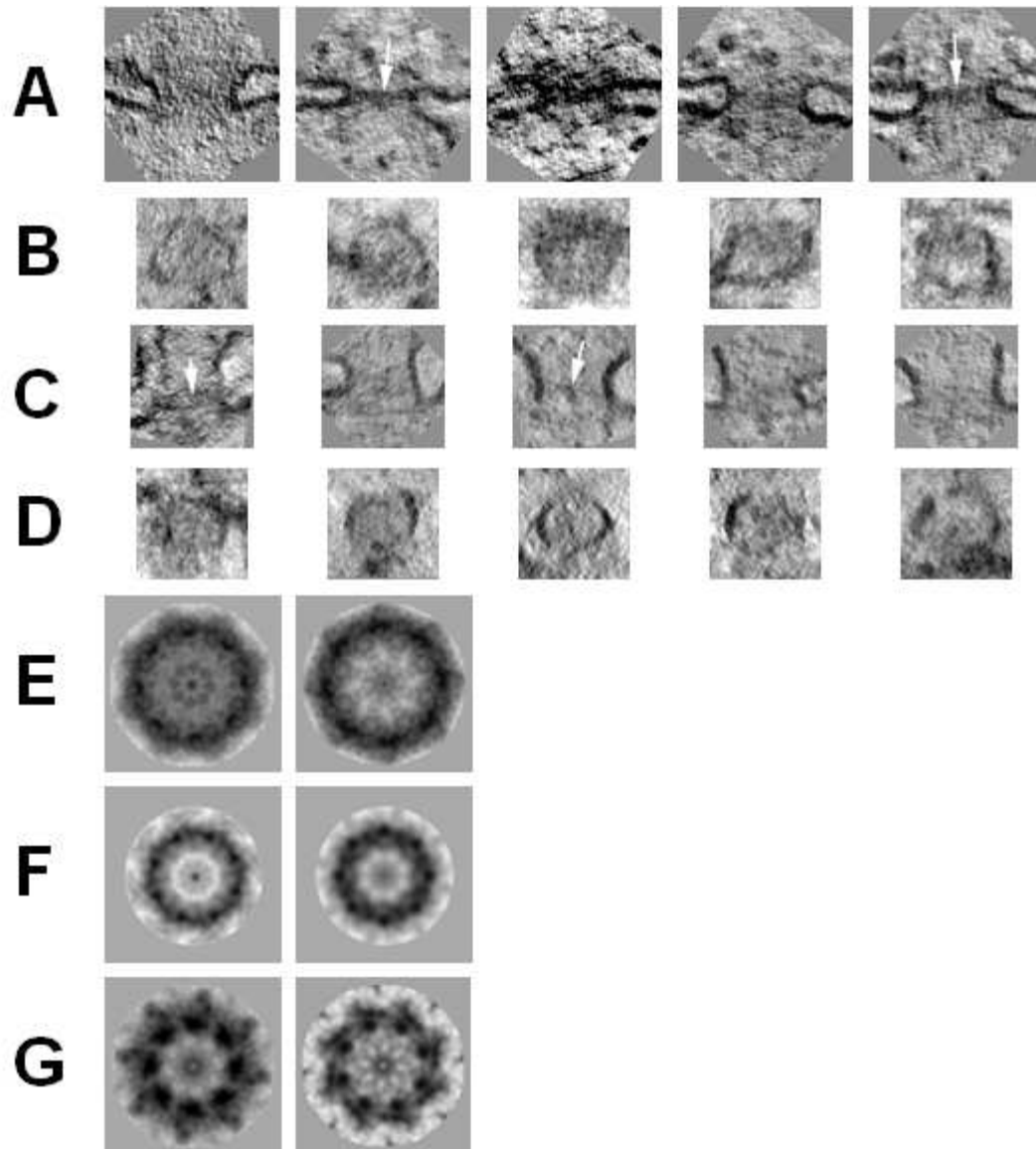


Figure 3.15: Visualization of nuclear pore and neck subvolumes. (A) Representative slices of nuclear pores from E18 torsinA KO nuclear envelopes. Slices are aligned parallel to the nucleocytoplasmic axis. Arrows point to a structure of the NPC visible in the slice, likely the luminal spoke ring (LSR). (B) The same nuclear pore subvolumes rotated so the slice is aligned perpendicular to the nucleocytoplasmic axis at about the plane of the LSR. (C) Representative slices from bleb subvolumes of E18 torsinA KO nuclear envelopes. Slices are aligned parallel to the nucleocytoplasmic axis. The nucleoplasm lies on the bottom of each slice. Arrows point to what appears to be a LSR. (D) The same bleb subvolumes rotated so the slice is aligned perpendicular to the nucleocytoplasmic axis. (E) Averaged subvolumes of E18 nuclear pores (left) and necks (right). (F) Averaged subvolumes of HEK nuclear pores (left) and HEK necks (right). (G) Averaged subvolumes of DIV5 nuclear pores (left) and DIV5 necks (right). The averages appear to be from structures of the NPC ranging from the cytoplasmic ring to the nuclear ring, explaining differences across cell types.

3.8. Inner Bleb Structures

A group of unexpected structures were identified within E18 torsinA KO blebs. These structures, given the name inner bleb structures (IBS), were not seen in reconstructions of HEK or DIV5 blebs. The majority of IBSs exhibited a rod-shaped morphology. A variety of views from IBS surface renderings are shown in Figure 3.16. Similar to blebs and vesicles, IBSs possess a strong linear surface area to volume relation as seen in Figure 3.17. The slope of the linear regression line fit to the data (the S-V ratio) is 129.57, a value much higher than that of blebs and vesicles. The R^2 statistic for the fit is 0.977, indicating a very close fit.

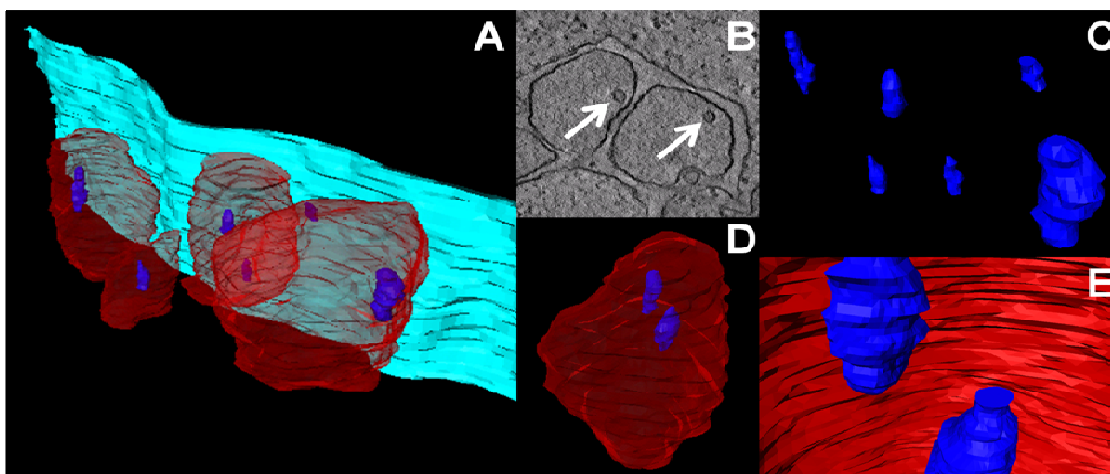


Figure 3.16: Different views of inner bleb structures. (A) Surface rendering of transparent blebs (red) showing IBSs (blue) in their interior. The ONM is omitted to provide a clear depiction of the blebs. (B) Single slice from a reconstruction depicting two blebs with arrows pointing to IBSs inside of each. (C) Subset of five IBSs depicting structural variety. (D) A single transparent bleb with two IBSs in its interior. (E) View of two IBSs from the inside of a bleb to show depth and perspective.

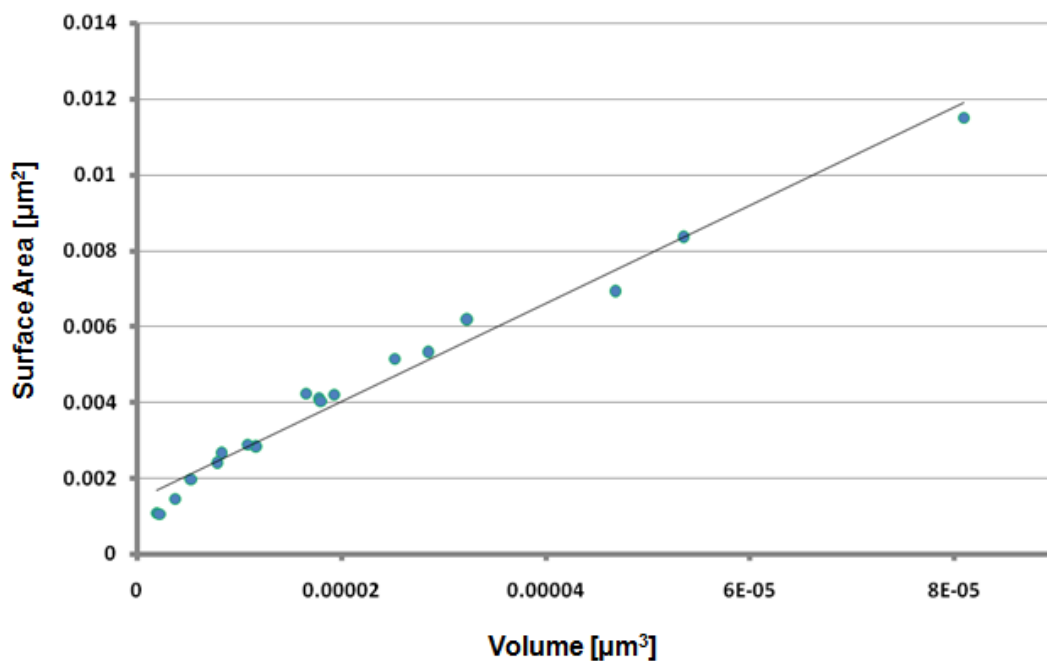


Figure 3.17: IBSs exhibit a linear surface area to volume relationship. The slope of the regression line, the SV ratio, is 129.57. The R^2 statistic for the fit is .9773.

An adaptation of the text and figures in this chapter will be submitted for publication with the following co-authors: Guy Perkins, Mark Ellisman, William Dauer, Guido Gaietta, and Tom Deerinck. The thesis author was the primary contributor to the work of this chapter.

CHAPTER 4: DISCUSSION

The Δ GAG torsinA mutation present in a large percentage of DYT1 dystonia patients leads to the formation of blebs and perinuclear vesicles that appear to bud from the inner nuclear membrane in a mouse model (Goodchild et al., 2005). TorsinA knockout and mutation knock-in mice both exhibit NE blebs, indicating that the Δ GAG mutation is a loss-of-function allele. Bleb appearance following the loss of WT torsinA is neural-specific, a fact that imitates the neural specificity of dystonia despite the fact that torsinA is ubiquitously expressed. Although its exact function is currently unknown, torsinA is a member of the AAA+ family of ATPases and likely functions by unfolding or disassembling protein complexes on its substrate.

The perinuclear luminal domain of LAP1, a transmembrane protein of the nuclear lamina that lines the inner nuclear membrane, is a substrate of torsinA (Goodchild and Dauer, 2005). Therefore, it was hypothesized that the appearance of the INM bleb phenotype in torsinA knockout and mutation knock-in mice may be due to an altered interaction between mutant torsinA and LAP1. The fact that the Δ GAG mutation causes a dramatic re-localization of torsinA from the lumen of the endoplasmic reticulum to the perinuclear space where it is able to act on LAP1 lends further credence to this hypothesis (Goodchild and Dauer, 2004). LAP1 belongs to the group of lamina-associated polypeptides that have been shown to bind lamins *in vitro* (Gruenbaum et al., 2005). The lamins are a set of type-V intermediate filament proteins that constitute the main components of the nuclear lamina. Recent evidence suggests the lamins play a role in a plethora of critical cellular activities including chromatin organization and nucleus reassembly following mitosis (Lopez-Soler et al.,

2001). Additionally, the lamins play an important role in nuclear pore architecture, serving to anchor the nuclear pore complex at the nucleoplasmic side of the INM (Stoffler et al., 1999).

In this thesis, the structural characteristics of blebs and vesicles were studied in torsinA KO neurons (E18), LAP1 KO neurons (DIV5), and LAP1 KO non-neurons (HEK). The fundamental goal was to explore the process of bleb and vesicle genesis in each condition. To achieve this, electron tomography was employed to generate three-dimensional reconstructions of segments of nuclear envelope demonstrating the bleb and vesicle phenotype. Single particle analysis was used to generate representative averages of pores and bleb necks from each condition. Pore and neck diameters and density were determined. Surface renderings of blebs and vesicles were used to determine their surface area and volume. The results of these experiments and their impact on the understanding of dystonia pathogenesis are discussed in detail in the following sections.

4.1. Normal Nuclear Structure Requires a WT torsinA & LAP1 Interaction

The presence of nuclear blebs and vesicles in all three cell types indicates that it is the disruption of a normal interaction between torsinA and LAP1 that leads to these ultrastructural abnormalities. E18 (torsinA KO), HEK (LAP1 KO), and DIV5 (LAP1 KO) cells all display similar trends between neck and pore diameter, neck and pore density, and S-V ratio. Since almost identical phenotypic manifestations are seen in neural cells lacking torsinA and neural cells lacking LAP1, it appears that it is the lack of interaction between these two partners that leads to bleb and vesicle formation. Interestingly, non-neural HEK LAP1 KO cells exhibited blebs and vesicles

whereas blebs and vesicles caused by torsinA loss are neural-specific (Goodchild et al., 2005).

4.2. Bleb Genesis

The structural evidence presented in this study strongly supports the hypothesis that NE blebs bud off from the INM as true vesicles and originate from nuclear pores. Blebs remain temporarily attached to the inner nuclear membrane via a membranous neck of variable length before budding off into the perinuclear space. These findings not only have great implications for dystonia pathogenesis but may suggest a greater cellular role for torsinA than was previously believed to exist. In the following subsections, the results leading to these conclusions are discussed in detail.

4.2.1. Pore and Neck Diameters Indicate that Blebs Bud from Pores

As reported in Figure 3.14, the maximum pore and neck diameters for each cell type studied are significantly similar, lending support to the hypothesis that blebs bud from a structure similar in size to a nuclear pore. An interesting finding was that pore diameters vary by a decent amount across cell types even though pores and necks within the same cell type are statistically similar. DIV5 pores possess the greatest diameter (95.11 ± 3.89 nm) and E18 pores are the smallest (67.81 ± 2.80 nm). While the overall three-dimensional assembly of the NPC is very well conserved among species, linear dimensions such as diameter are known to vary on a species by species basis (Fahrenkrog and Aebi, 2003). Therefore, the differences in pore diameter across each cell type studied could be due to differences in their sources: embryonic mouse cervical spinal cord tissue, a human embryonic kidney cell line, and

mouse neurons cultured *in vitro* for E18, HEK, and DIV5 cells, respectively. Additionally, the NPC is not perfectly cylindrical and its diameter varies across the length of the pore. In *dictyostelium discoideum* nuclei, the central pore has a diameter of roughly 60 nm while the entire NPC from one POM to the other has a diameter of about 125 nm (Beck et al., 2004). The diameter is smallest around the luminal spoke ring located at the center of the plane of the pore and increases in both the cytoplasmic and nucleoplasmic directions. Although measurements were made close to the center of the pore plane, the exact structures of the NPC were not readily discernible in subvolumes and measurements of other NPC features may have been made instead, a fact that would tend to make diameters larger than expected. However, this would result in only minor differences between measurements. It is most likely that the large differences in pore diameters across cell types are a result of their different sources.

4.2.2. Structural Similarity Between Pores & Necks

There is a high degree of similarity between the symmetrized neck and pore averages of Figure 3.15, adding additional support to the hypothesis that blebs bud from nuclear pores. Even though the number of subvolumes included in each average was small, the final averages compare very favorably in quality to those of Beck and colleagues, for which averages were created from sets with roughly 100 subvolumes each (Beck et al., 2004). The NPC possesses well known eight-fold symmetry, with the spoke as the fundamental symmetric unit. Each spoke is divided into two nearly identical halves joined at the center of the pore plane (Alber et al., 2007). The eight spokes are clearly visible in each symmetrized average of both

pores and necks (Figure 3.15). Additionally, the central channel or plug is visible at the center of each average. A comparison of the pore averages obtained to averages previously reported by Beck and colleagues indicates that the pores appear to be slices around the luminal spoke ring (LSR) or nuclear ring (NR). Since both of these structures are near the center of the plane of the pore, this conclusion is logical based on the fact that subvolumes were aligned at the center of each pore. A comparison between neck averages and published averages leads to a similar conclusion: neck averages also demonstrate strong eight-fold symmetry and appear to be slices from the LSR or NR. Additionally, both pore and neck averages compare favorably to a projection of nucleoporin mass density recently reported by Alber and colleagues (Alber et al., 2007).

4.2.3. Blebs Appear to Detach from the INM as Vesicles

Since ET reconstructions were performed on a preserved sample, the time dependent behavior of blebs and vesicles in three-dimensions could not be fully studied. Thus, it is not possible to definitively state if blebs bud off from their membranous connection to form perinuclear vesicles. However, the presence of similarly shaped perinuclear vesicles in the same region of the NE from which blebs are budding seems to support the hypothesis that blebs truly detach from the INM. A small portion of each bleb remains attached to the NE via a membranous connection of variable length; vesicles within the perinuclear space do not exhibit this connection. To test for a shared origin between blebs and perinuclear vesicles, a structural analysis on both was performed. Blebs and vesicles across all cell types have a very similar surface area to volume relationship, defined as the slope of the linear

regression line relating surface area and volume (Figure 3.13, Table 3.3). The high degree of correspondence between the surface area to volume ratios of blebs and vesicles strongly indicates the two share a similar origin. Of further interest is the fact that the S-V ratio for both blebs and vesicles is very similar across all cell types, indicating the possibility that both form by a process that is conserved regardless of cell type and species. This adds further support to the hypothesis that blebs bud from nuclear pores. Although the structural analysis presented is certainly not definitive, it does strongly suggest that blebs bud off of the INM to form true vesicles. Further experiments are necessary to reach a definitive conclusion.

4.3. Decreased NPC Density in Affected Nuclei

The nuclear pore density values reported in Table 3.4 as number of pores per μm^2 of INM are significantly below values reported in the literature. Beck and colleagues reported an NPC density of about $45/\mu\text{m}^2$ from cryoelectron tomograms of *dictyostelium* nuclei (Beck et al., 2004). Due to the dynamic nature of the nucleus and its importance in chromosome organization, the number of nuclear pores is known to vary greatly across the various stages of the cell cycle. In a study by Winey and colleagues, NPC density was found to peak during the S-phase of the cell cycle based on electron micrographs of *saccharomyces cerevisiae* nuclear envelopes (Winey et al., 1997). However, even during late anaphase when NPC density was found to be a minimum, the reported NPC densities are significantly greater than those reported in Table 3.4 for all three cell types. If neck density and pore density from Table 3.4 are summed for each reconstruction, the summed density still fails to approach those reported in the literature.

The process of bleb formation from the INM may provide an explanation for the decreased NPC density seen in affected nuclei. As previously discussed, INM blebs appear to bud from nuclear pores, pushing out the ONM in the process. The entire NPC in normal cells has a length of about 110 nm when viewed in slices parallel to the nucleocytoplasmic axis (Beck et al., 2004). Blebs seen in both torsinA and LAP1 KO nuclei were very large, pushing the INM and ONM apart by a size many times larger than this length. The force generated by the bleb pushing against the hemipores of the NPC is likely great enough to compromise NPC structure, leading to their destruction or partial incorporation into the bleb. Additionally, the force generated by one bleb pushing against the ONM may be great enough to disrupt and pull apart adjacent NPCs from which blebs are not budding.

In addition to the mechanical disruption of NPCs, a deeper molecular mechanism may exist to explain decreased NPC density in the cells studied. Although not reported in Table 3.4, density measurements were similarly low for reconstructions from regions of HEK nuclear envelope devoid of blebs. It is possible that these regions once possessed blebs that either broke down in the perinuclear space or fused with the ONM as vesicles. The previous presence of blebs would lead to disruption of NPCs followed by a return to normal NE dimensions following their exit from the system before new NPCs form. Alternatively, a molecular mechanism linking torsinA to NPC distribution may exist. The lamins have been shown to be critical in anchoring the NPC to the INM as well as in determining NPC distribution (Hutchinson, 2002). An altered interaction with mutant torsinA may cause an abnormal interaction between LAP1 and the lamins, leading to decreased NPC

stability. This hypothesis is supported by the fact that pore density is low in both torsinA and LAP1 KO cells.

4.4. The Rod-Shaped Morphology is Unique to torsinA Loss

Roughly one quarter of torsinA KO vesicles and blebs demonstrate a rod-shaped morphology while the rod-shape is absent in LAP1 KO nuclei (Figure 3.12). A survey of the literature revealed a lack of studies regarding the morphology of such vesicles. For any vesicle volume less than the volume of the sphere with an equivalent radius, the vesicle can theoretically assume an infinite number of shapes (Svetina and Zeks, 2002). However, only certain morphologies are seen experimentally, as was the case with this study. A variety of continuum models exist for the prediction of vesicle shape. The most common model, proposed by Evans and Skalak, depicts the vesicle phospholipid membrane as a thin elastic sheet for which vesicle shapes are related to the mechanical properties of their membranes (Evans and Skalak, 1980). The shapes assumed by vesicles are thought to correspond to the configuration with the minimum membrane bending energy (Deuling and Helfrich, 1976). Despite these theoretical models, a method for predicting the three-dimensional morphology of vesicles budding from the INM does not currently exist. However, the presence of rod-shaped vesicles in only torsinA KO nuclei indicates that torsinA may play an additional role in vesicle formation independent of its interaction with LAP1. The lack of torsinA may change the mechanical properties of the INM, allowing for the formation of rod-shaped vesicles.

4.5. Inner Bleb Structures are Unique to torsinA Loss

Similar to the rod-shaped vesicle morphology, IBSs are seen only in torsinA KO neurons. Inner bleb structures are found in blebs as well as vesicles, a fact that lends further support to the belief that blebs bud off as true vesicles. The volume of the IBS is about an order of magnitude greater than that of a ribosome (Malhotra et al., 1998; Verschoor et al., 1997). These structures possess a range of volumes and surface areas as do blebs and vesicles (Figures 3.13 and 3.17), indicating that they are variable in size and likely not a consistent structure such as a ribosome. IBSs possess a strongly linear surface area to volume relationship, a trend that is again shared with blebs and vesicles. However, the S-V ratio for IBSs is roughly 3-4 times greater than that of blebs and vesicles. As demonstrated in Figure 3.16, IBSs demonstrate a rod-shaped morphology and are found in blebs and vesicles of all morphologies.

Besides the details discussed, nothing is known about these structures and their appearance was surprising. Their dense and prominent appearance in slices of reconstructions indicates that they may be membrane-bound. The rod-shaped morphology, linear surface area to volume relationship, and appearance in only torsinA deficient neurons all seem to suggest that the IBSs may be a subset of the rod-shaped vesicles found only in torsinA KO cells. Similar to the rod-shaped vesicle morphology, their appearance is likely due to torsinA deficiency rather than an altered interaction between torsinA and LAP1. The importance of these structures must be characterized in future studies, as they are likely to be important to understanding torsinA function at the nuclear envelope.

4.6. A Possible Role for Perinuclear Vesicles in DYT1 Pathogenesis

Although the analysis of blebs and vesicles performed in this study was of a purely structural nature, it is possible to speculate on the linkage between vesicles and DYT1 dystonia pathogenesis. As discussed in Section 1.1.2, there is strong evidence to link dystonia to abnormalities in dopamine neurotransmission. Due to its apparent association with dystonia pathogenesis, it is possible that perinuclear vesicles may act to disrupt dopamine transmission. Vesicular monoamine transporter 2 (VMAT2) is a membrane protein synthesized in the ER and found primarily in the CNS (Fukui et al., 2007). VMAT2 is responsible for loading cytoplasmic monoamines into secretory vesicles and its abundance appears to have a strong correlation with a cell's ability to secrete dopamine. Normal dopamine transmission requires its sequestration into secretory vesicles to protect against cytosolic degradation. In a recent study, VMAT2 heterozygote knockout mice were found to demonstrate a lower vesicular dopamine content and dramatically reduced striatal extracellular dopamine (Reveron et al., 2002). Decreased striatal dopamine has been found to lead to the development of dystonia in baboons, demonstrating a potential link between VMAT2 deficiency and dystonia pathogenesis (Perlmutter et al., 1997).

In a recent study, VMAT2 was found to colocalize and accumulate with torsinA in perinuclear inclusions of cultured human SH-SY5Y neuroblastoma cells (Misbahuddin et al., 2005). Since torsinA is a AAA+ protein with a possible chaperone function, its Δ GAG mutation may facilitate the concomitant movement of VMAT2 to the NE. The inclusion of VMAT2 into perinuclear vesicles may lead to its sequestration within the perinuclear space, preventing its transport to the cytosol via the normal secretory pathway (Misbahuddin et al., 2005). This would in turn reduce or

alter cellular dopamine release, a consequence that may lead to dystonia. An additional protein, snapin, has been shown to be recruited to perinuclear inclusions in ΔE -torsinA SH-SY5Y cells (Granata et al., 2008). Snapin interacts with components of the SNARE complex whose primary role is to mediate fusion of vesicles with cell membranes (Ilardi et al., 1999). Similar to VMAT2, the sequestration of snapin within perinuclear vesicles may lead to a decrease in normal secretory processes. Additionally, the colocalization of ΔE -torsinA and snapin may indicate that torsinA is a regulatory protein in the process of vesicle fusion and recycling.

The hypothesis that proteins critical for the secretory process and dopamine release may become trapped in perinuclear vesicles corroborates well with the data presented in this study. The incorporation of the predominantly CNS-located protein VMAT2 in vesicles may help to explain the neural specificity of the dystonia phenotype despite the appearance of vesicles in non-neurons. The stereotyped formation of blebs from a single nuclear pore implicates a standardized process by which proteins such as VMAT2 and snapin may be incorporated into vesicles. Decreased nuclear pore density in affected nuclei may be a product of the transient process of sequestration of key proteins into perinuclear vesicles.

4.7. Advantages of Single-Particle Analysis with Electron Tomograms

The use of single-particle analysis (SPA) combined with electron tomography presents a number of advantages over traditional SPA. Traditional SPA is used to build the three-dimensional structure of an object by estimating the projection angles of a large number of randomly oriented two-dimensional electron micrographs of the object (Wang and Sigworth, 2006). The protocol typically involves dividing the images

into clusters, estimating the projection angle of each cluster, and building cluster averages. The major drawback of traditional SPA is that the resulting averages are highly sensitive to how the total set is divided into clusters as well as the choice of reference image used for each cluster. These processes are not automatic and thus depend heavily on the user and may not be repeatable (Asai et al., 2000). Additionally, the process of generating a three-dimensional structure from two-dimensional micrographs involves a great number of assumptions and extrapolations. SPA using electron tomography circumvents these problems by utilizing subvolumes from three-dimensional reconstructions, eliminating much of the guesswork.

Another advantage of combining SPA with electron tomography lies in the fact that subvolumes originate from tomograms of an intact nucleus, allowing for SPA to be performed *in situ*. A number of previous studies of NPC three-dimensional structure using SPA utilized NPCs extracted from the nuclear envelope by treatment with detergent (Stoffler et al., 2003; Yang et al., 1998; Akey and Radermacher, 1993). Recent estimates indicate that the NPC is a very delicate structure consisting of at least 456 individual protein molecules, so detergent extraction is likely to cause irreversible damage its structure (Alber et.al, 2007). Additionally, detergent-extracted NPCs assume a preferred orientation when deposited on EM grids, leading to anisotropic sampling (Grunewald et al., 2002). However, boxed NPCs from electron tomograms of an intact nucleus assume random spatial orientations that cover all Eulerian angles and lack a stereotyped orientation (Beck et al., 2004). Thus, single-particle averages from electron tomograms are more likely to approximate the true structure of the NPC.

4.8. Conclusions

The appearance of perinuclear blebs and vesicles in both torsinA and LAP1 KO neural cells indicates that it is an abnormal interaction between these two interacting partners rather than just simple torsinA loss that leads to structural abnormalities of the nucleus in dystonia models. The apparent involvement of the nuclear lamina in DYT1 pathogenesis implies a possible similarity between dystonia and the laminopathies, a diverse set of disorders involving the lamina. The appearance of blebs and vesicles in LAP1 KO non-neural cells but lack of this phenotype in torsinA KO non-neural cells suggests that torsinA plays a different role in the nuclear envelopes of neural and non-neural cells.

Single-particle analysis and electron tomography indicate that perinuclear vesicles bud from a single nuclear pore in all conditions studied. This process likely pushes apart the NPC, leading to the destruction of the NPC regions close to the ONM. The fact that vesicles appear to bud from a single pore suggests a conserved process of vesicle genesis independent of cell type or species. Thus, the abnormal interaction between mutant torsinA and LAP1 is likely the molecular mechanism leading to vesicle formation. Since torsinA is highly conserved amongst eukaryotes, the stereotyped process of vesicle formation regardless of cell type is logical. The decrease in NPC density of both torsinA and LAP1 KO cells provides further support to this claim. Decreased pore density may be due to mechanical disruption of the nuclear envelope caused by the force generated from blebs pushing out against the ONM or may be due to a molecular mechanism linking LAP1 to the lamins and NPC anchoring. Finally, the similar surface area to volume ratio of vesicles across cell types lends further support to the hypothesis of a stereotyped vesicle formation.

Although the analysis performed in this study was of a purely structural nature, it is possible to speculate on the linkage between vesicles and DYT1 dystonia pathogenesis. VMAT2 and snapin, proteins involved in dopamine transmission and vesicle fusion, respectively, have been shown to aggregate with mutant torsinA in perinuclear inclusions. The possible inclusion of a protein linked to cellular dopamine transmission may provide a key connection between the vesicle phenotype and dystonia pathogenesis. A variety of studies have linked dystonia to abnormal dopamine neurotransmission. The sequestration of VMAT2 within perinuclear vesicles may inhibit its function of cytosolic vesicle formation and decrease dopamine transmission, leading to abnormal motor patterns and dystonia.

The specificity of the rod-shaped vesicle morphology and inner bleb structures to torsinA KO cells may indicate a neural role for torsinA in perinuclear vesicle formation that is independent of its interaction with LAP1. Since these discoveries were unexpected, little can be said about their possible roles in dystonia pathogenesis without further experiments. However, the fact that their appearance is specific to torsinA loss indicates a greater cellular function for torsinA in the maintenance of nuclear envelope architecture and mechanics.

The findings of this thesis serve to further the understanding of dystonia pathogenesis and torsinA function. The mechanical and molecular mechanisms behind perinuclear vesicles and their genesis are likely critical to understanding the pathophysiology of dystonia. This research presents the first use of electron tomography to study dystonia as well as the first structural characterization of perinuclear vesicles. Additionally, it is the first application of single-particle analysis to study vesicle genesis. The results of this thesis provide a greater understanding of

dystonia pathogenesis and torsinA function and serve to direct future research in these fields.

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