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The gene responsible for Dyggve-Melchior-Clausen syndrome encodes a novel peripheral membrane protein dynamically associated with the Golgi apparatus

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Short title: Dym, a novel disease-associated Golgi protein

Key words: DMC syndrome, Dymeclin, myristoylation, Golgi apparatus, FRAP, nocodazole

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Abstract

Dyggve-Melchior-Clausen dysplasia (DMC) is a rare inherited dwarfism with severe mental retardation due to mutations in the *DYM* gene which encodes Dymeclin, a 669-aminoacid protein of yet unknown function. Despite a high conservation across species and several predicted transmembrane domains, Dymeclin could not be ascribed to any family of proteins. Here we show, using *in situ* hybridization, that *DYM* is widely expressed in human embryos, especially in the cortex, the hippocampus and the cerebellum. Both the endogenous and the recombinant protein fused to GFP co-localized with Golgi apparatus markers. Electron microscopy revealed that Dymeclin associates with the Golgi apparatus and with transitional vesicles of the reticulum-Golgi interface. Moreover, permeabilization assays revealed that Dymeclin is not a transmembrane but a peripheral protein of the Golgi apparatus as it can be completely released from the Golgi after permeabilization of the plasma membrane. Time lapse confocal microscopy experiments on living cells further showed that the protein shuttles between the cytosol and the Golgi apparatus in a highly dynamic manner and recognizes specifically a subset of mature Golgi membranes. Finally, we found that *DYM* mutations associated with DMC result in mis-localization and subsequent degradation of Dymeclin. These data indicate that DMC results from a loss-of-function of Dymeclin, a novel peripheral membrane protein which shuttles rapidly between the cytosol and mature Golgi membranes and point out a role of Dymeclin in cellular trafficking.

Introduction

Dymeclin is a protein recently identified as the translation product of the FLJ20071/FLJ90130/Dym gene (*DYM*) which mutations are responsible for a severe autosomal-recessive skeletal dysplasia associated with mental delay, Dyggve-Melchior-Clausen syndrome (DMC, MIM#223800) and a clinical variant without mental impairment, Smith-McCort syndrome (SMC, MIM#607326), (1, 2). DMC/SMC skeletal features consist of a short trunk dwarfism with particular radiological features, severe proximal limb shortening and microcephaly associated with a peculiar facial dysmorphism (3). Mental retardation is consistently observed in DMC with an average IQ between 25 and 65. Children are often hyperactive, display autistic features and do not speak. Both skeletal and mental features are progressive in DMC, and the phenotype often leads to severe orthopedic complications (3).

Dymeclin is a 669-aminoacid protein which does not belong to any identified protein family. Except for five or six putative transmembrane domains and a N-terminal myristoylation site, search for predictive structural and/or functional motives failed to provide any clue as to the function of Dymeclin (1, 2). Most of the mutations identified in *DYM* predict the generation of a truncating product; however, a few frameshift mutations in the last exon predicting an elongated protein, two complex genomic duplications resulting in exon repetition, and two missense mutations have also been reported (1, 2, 4). At the ultrastructural level, DMC cells disclose enlarged endoplasmic reticulum (ER) network and accretion of intracytosolic membranous vesicles (2, 3, 5, 6). It has therefore been proposed that Dymeclin may be an integral membrane protein of the ER possibly involved in the transport of intracellular compounds (7). Because the DMC phenotype resembles that of type IV mucopolysaccharidosis (Morquio disease), a lysosomal disorder due to either N-acetylgalactosamine-6-sulfatase or β -galactosidase deficiency, it has also been hypothesized that Dymeclin has a role in intracellular digestion of proteins. However, the cellular phenotype of DMC rather evokes abnormal storage and/or membrane trafficking involving specifically the endoplasmic reticulum network. It therefore clearly differs from that of the Morquio disease in which ultrastructural anomalies involve lysosomes. In addition, biochemical analyses failed to reveal any enzymatic deficiency or accumulation of specific substrate in DMC cells (3).

In order to obtain additional clues about Dymeclin function, we studied the expression pattern of the *DYM* gene in human embryonic and foetal tissues using *in situ* hybridization and

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3 investigated the sub-cellular localization and the dynamics of the protein in living cells. We
4 show that *DYM* transcripts are widely expressed throughout human development and that
5 Dymeclin is not an integral membrane protein of the ER, but rather a peripheral membrane
6 protein dynamically associated with the Golgi apparatus. Using different mutations previously
7 identified in DMC and SMC patients, we show that all mutated proteins are mis-localized and
8 subsequently degraded except for one particular mutation reported in SMC.
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18 Results

19 *DYM* transcripts are widely expressed during human development

20 Northern blot analysis of mRNA derived from human tissues previously showed that *DYM*
21 has two transcripts (3.1 and 5.6 kb) abundantly expressed in foetal brain (2). Using two non-
22 overlapping riboprobes selected in either the 5'-half of *DYM* (probe A, 0.85 kb) or its 3'-half
23 (probe B, 0.5 kb, not shown), we revealed the same two RNA species, thus confirming their
24 specificity. We also show that *DYM* is abundantly expressed in primary chondrocytes and
25 osteoblasts (Fig. 1A). Because mutations in *DYM* specifically affect the cognitive
26 development of children and their bone growth in a progressive manner, we questioned
27 whether *DYM* expression was specific to nervous and osseous tissues or if it was more widely
28 expressed during human development. To address this question, a human multiple tissue
29 expression array was probed with probe A. Consistent with a wide range of expression, *DYM*
30 transcripts were detected in most adult tissues (Fig. 1A). Highest levels of expression were
31 seen in the cerebellum, kidney, lung and stomach, but in the heart and the pancreas as well.
32 However, very low or no expression was observed in the spleen, thymus, oesophagus, bladder
33 and thyroid gland. *DYM* was also expressed in a variety of human cell types, including HeLa,
34 SaOs2 and skin fibroblasts and was detected in murine ES cells (Fig. 1A).
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As to investigate the spatio-temporal expression pattern of *DYM* during human development,
we performed in situ hybridization on human embryonic and foetal tissues at different
developmental stages, Carnegie Stage 17 (CS17, 40 day embryo), Carnegie Stage 19 (CS19,
47-48 day embryo), 17 and 22 weeks of gestation foetus. At CS17, expression of *DYM* was
slightly detectable in numerous structures of the embryo, including the neural tube, the cranial
nerve nuclei, the mandible, the stomach, and the limb bud, but no structure showed a
particularly marked signal (data not shown). Hybridization at CS19 confirmed the widespread
expression of *DYM* with a higher expression in the germinative zone of the prosencephalon,
mesencephalon and cerebellum anlage (Fig. 1B, a,b,c) but also in mesenchymal condensations

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3 of the lung and intestinal epithelia (Fig. 1B, a,d,e,f,g). Surprisingly enough, no specific signal
4 could be detected in the vertebrae of the CS19 embryo. Specific expression in the cerebellum
5 was confirmed at later stages. In the 17 week cerebellum, *DYM* was mostly expressed in the
6 external granule cell layer, the germinative zone which gives rise to the internal granule
7 neurons between 16 and 40 weeks (Fig. 1B, h,i,j,k). At 22 weeks of age, *DYM* was highly
8 expressed in the external granule layer, in the Purkinje neurons and in the internal granule
9 layer (Fig. 1B, p,q,r,s). At both 17 and 22 weeks, specific labelling was observed in the
10 neocortical plate and corresponding ventricular zone (germinative zone) as well as in the
11 hippocampus (Fig. 1B, l,m,n,o and t,u,v,w). No specific labelling was detected in areas of
12 neuronal migration at any stage.

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14 Together, these data show that human *DYM* expression is not restricted to nervous and
15 osseous tissues but is also expressed in most embryo-foetal and adult tissues.

26 **The *DYM* gene product, Dymeclin, is a Golgi-associated protein**

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28 The coding sequence of *DYM* is predicted to produce a 669-aminoacid protein we have
29 previously named Dymeclin (2). As to characterize Dymeclin, we generated a polyclonal
30 antiserum against a 16-aminoacid peptide selected in the amino-terminal half of the protein in
31 a region conserved between mouse and human. The sub-cellular localization of Dymeclin was
32 assessed in HeLa cells by immunofluorescence. As shown in Figure 2A, we found
33 endogenous Dymeclin in the cytosol but it was predominantly detected as a perinuclear
34 pattern reminiscent of the Golgi complex. When cells were double-labelled for Dymeclin and
35 GM130, a marker of the *cis*-Golgi, an extensive overlap was observed. Specificity of our
36 antibody could be checked in immunofluorescence using a mis-localized mutant of Dymeclin
37 (Fig.2A), although we did not detect a stronger Golgi labelling upon over-expression of
38 exogenous Dymeclin, suggesting that Golgi localization may be saturable. We were also able
39 to immunoprecipitate the recombinant protein fused to GFP at its carboxy-terminus, Dym-
40 GFP (~105kDa, Fig.2B). However, our antibody did not raise specific signal in western blot.
41 Importantly, GFP-tagged Dymeclin was similarly found on Golgi elements. HeLa cells were
42 transfected with Dym-GFP and stained for immunofluorescence using various markers of the
43 *cis*-Golgi (GM130 and Giantin), the *trans*-Golgi/*trans*-Golgi Network (Galactosyl Transferase
44 and TGN46) and of the endoplasmic reticulum (PDI, Fig. 4B). Like the endogenous protein,
45 recombinant Dymeclin strongly co-localized with markers of the *cis* and *trans*-Golgi and was
46 closely apposed to the TGN marker (Fig. 3A). By contrast, Dymeclin did not co-localize with
47 markers of the endoplasmic reticulum (Fig. 4B). Similar results were obtained using an
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3 amino-terminal GFP-tagged Dymeclin (GFP-Dym) or a myc-tagged Dymeclin (data not
4 shown). Immuno-electron microscopy was used to further analyze the location of Dymeclin
5 within the Golgi apparatus of HeLa cells. Immuno-gold labeling of Dym-GFP on cryosections
6 confirmed the presence of Dymeclin near the membranes of the Golgi apparatus and on
7 vesicular structures of transfected cells (Fig. 3B).
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11 No conserved domains or signal peptides could be predicted from the Dymeclin primary
12 sequence, except for a putative N-terminal myristoylation site “MGSNSSR” (2). Protein N-
13 myristoylation corresponds to the covalent attachment of a myristate, a 14-carbon saturated
14 fatty acid, to the N-terminal glycine of eukaryotic proteins and promotes, in many cases, a
15 weak and reversible membrane association (8). Because the MGSNSSR site found in
16 Dymeclin was N-terminal and included a glycine residue in position +2, we tested whether
17 this motif could be myristoylated *in vitro*. Carrying out *in vitro* translation of wild type and a
18 G2A mutant in the presence of radioactive myristate, we could show that Dymeclin can be
19 myristoylated on the G at position +2 (Fig. 4A). We then asked whether myristoylation was
20 essential for Dymeclin to localize to the Golgi apparatus. The mutant G2A was expressed
21 fused to GFP in HeLa cells. The cells were fixed 24 hours after transfection and stained for
22 immunofluorescence with the endoplasmic reticulum marker PDI. Although we observed a
23 slight increase in cytosolic localization of Dym-G2A-GFP, it clearly localized on the Golgi
24 apparatus (Fig. 4B). Similarly, we observed that amino-terminal tagging of Dymeclin, while it
25 prevents myristoylation, does not prevent Dymeclin from binding to the Golgi apparatus (data
26 not shown). Altogether, these results indicate that Dymeclin is a Golgi-associated protein and
27 that myristoylation is dispensable for Dymeclin binding to the Golgi apparatus.
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44 **Dymeclin is a peripheral Golgi protein**

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46 Based of hydrophobic domain analysis, it has been proposed that Dymeclin may be a multi-
47 spanning transmembrane protein (7). To address this question, we used a permeabilization
48 assays in living cells adapted from a protocol described by Lorenz et al. (9). HeLa cells stably
49 expressing mCherry-Histone were transfected with GFP-tagged Dymeclin and observed by
50 fluorescent microscopy. Cells were then treated by low concentration of digitonin to
51 permeabilize the plasma membrane and observed again. While Dym-GFP was present on the
52 Golgi apparatus and in the cytosol before treatment, it completely disappeared after 1 min
53 upon opening of the cells (Fig. 5). We compared Dymeclin behavior to ARF1 and GRASP65
54 behavior, two myristoylated proteins associated with the Golgi apparatus. Before treatment,
55 Dymeclin, GRASP65 and ARF1 display very similar localization (Fig. 5 a,c,e). However, the
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3 Golgi apparatus was still weakly stained by GRASP65 and ARF1 1 minute after
4 permeabilization (Fig. 5 e,f) suggesting that the association of ARF1-GFP and GRASP65-
5 GFP with the Golgi apparatus is more stable than that of Dymeclin. Because Dymeclin was
6 more rapidly released from the Golgi apparatus than two well known peripheral Golgi
7 proteins that dynamically exchange with the cytosol (10 , 11), we concluded that Dymeclin is
8 not a transmembrane protein but is a cytosolic protein which can be recruited to the Golgi
9 apparatus.
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18 **Dymeclin rapidly shuttles between the Golgi complex and the cytosol**

19 Because Dymeclin behaved as a peripherally associated Golgi protein and was also present in
20 the cytosol, we next examined the dynamics of the protein in living cells. HeLa cells were
21 transfected with GFP-tagged Dymeclin and fluorescence recovery after photobleaching
22 (FRAP) experiments were performed on living HeLa cells 24 hours after transfection. GFP-
23 tagged ARF1 and GRASP65 were used as references for Golgi-associated myristoylated
24 proteins. We measured the fraction of these proteins which is associated to the Golgi
25 apparatus (Fig. 6A). As reported previously (10), we found that $16.5 \pm 4\%$ of ARF1 is Golgi-
26 associated. We obtained very similar values for Dymeclin ($13.6 \pm 3.9\%$) while a larger pool
27 was measured for GRASP-65 ($33.2 \pm 10.8\%$). The recovery of fluorescence was quantified
28 for the three proteins after correction of the photobleaching resulting from the acquisition and
29 subtraction of the background. The quantifications were performed on 3D and 2D images and
30 gave similar results (data not shown). We therefore decided to work with 2D images in order
31 to increase the frequency of acquisition. The normalized intensity measured for 30 seconds
32 after FRAP for all proteins was plotted on the same graph (Fig. 6B). When Golgi-associated
33 Dym-GFP was photobleached, fluorescence recovered to the Golgi area in less than twenty
34 seconds (Fig. 6D and sup. Movie 1); this recovery is faster than that of the two reference
35 proteins (Fig. 6B). We calculated the mean half time of recovery and confirmed that Dym-
36 GFP recovers more rapidly than the two other proteins with a half time of 2.8 ± 0.9 seconds
37 (Fig. 6C). Arf1-GFP, which is known to be a fast shuttling protein (10 , 11) displayed a half-
38 time recovery of 7.1 ± 2.6 seconds while GRASP65-GFP had a slightly slower dynamics with
39 a half-time of 12.4 ± 4.5 seconds. These results demonstrate that Dymeclin is a highly mobile
40 protein as the entire Golgi pool can be exchanged in less than 20 seconds.
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Dymeclin specifically binds to a subset of the Golgi apparatus upon nocodazole treatment

We have shown in the past that depolymerization of microtubules by a nocodazole treatment reveals the presence of two separate Golgi pools (12). Most of Golgi-associated proteins (like GM130 and GalT) are relocated on the newly formed mini stacks that appear after microtubules disassembly whereas one protein, Giantin, is found only on the old, pre-existing Golgi complex that keeps its juxta-nuclear localization. This may be due to the fact that Giantin being a very large transmembrane protein is recycling very slowly to the newly formed mini-stacks. In order to test whether Dymeclin recognizes a particular set of Golgi stacks, HeLa cells expressing Dym-GFP were left untreated or incubated for 90 minutes in the presence of nocodazole, before being fixed and stained for GM130 and Giantin and observed by immunofluorescence. The three proteins strongly co-localized in control conditions (Fig. 7A, top line). After nocodazole treatment, and as reported before (12), GM130 was present on the mini stacks dispersed through the cytoplasm whereas Giantin was still located on “old” Golgi complexes that were present before nocodazole addition and that are still located in the pericentriolar region (Fig. 7A, bottom line). Surprisingly, Dym-GFP was present both as a soluble pool and on the “old” Golgi but not on the newly formed mini stacks. This result was very surprising as we have shown that Dym-GFP shuttles very rapidly between the Golgi apparatus and the cytosol and suggested that Dym-GFP specifically recognizes a subset of the Golgi apparatus. To test this hypothesis, we followed the dynamics of Dym-GFP after nocodazole treatment in living HeLa cells by FRAP. Cells were co-transfected with Dym-GFP and mCherry-GM130 and treated with nocodazole 24 hours after transfection (Fig. 7B). As expected, GM130 was found on dispersed dots in the cytoplasm while Dymeclin was preferentially located in a subset of cellular structures (see the GM130-positive element indicated by an arrowhead in Fig. 7B). Dym-GFP was photo-bleached and its recovery analyzed. White arrows in Figure 7B indicate the bleached region. Dym-GFP fluorescence had already largely recovered after 9 seconds (top line, Dym-GFP) whereas mCherry-GM130 did not recover during the time of acquisition (middle line, mCh-GM130). Therefore, Dymeclin specifically and dynamically recognizes a subset of the Golgi apparatus while it ignores other Golgi stacks, a behavior revealed in the absence of microtubules.

DMC mutations but not the E87K SMC mutation result in Dymeclin mis-localization and subsequent protein degradation

Most of the genetic mutations which have been identified in DMC patients are stop mutations, insertions and deletions causing frameshift or splice mutations that predict protein truncation (3). However, one missense mutation (N469Y) has been identified in some DMC patients while another one (E87K) has been associated with the SMC variant with no mental retardation (1, 2). Since mental retardation is variable in DMC (ranging from mild to severe), and totally absent in SMC, we asked whether the consequence of *DYM* mutations on the protein could differ between mutations. To this end, three nonsense mutations (R204X, Q483X and K616X) and the two missense mutations (N469Y and E87K) were introduced through site-directed mutagenesis in GFP-Dym. Following over-expression of these constructs in HeLa cells, the localization of the five mutant proteins was analyzed and compared to that of wild-type GFP-Dym and GM130. Although GFP fluorescence was globally found very weak in cells transfected with DMC mutations, numerous small aggregates were observed in many cells in a pattern suggestive of protein degradation (Fig. 8A). However cells transfected with the E87K mutation still displayed a pattern similar to that of the wild-type Dymeclin. Therefore, only DMC mutations resulted in a mis-localization and aggregation of Dymeclin. Since ubiquitin-rich cytoplasmic inclusion is linked to the pathogenesis of many diseases, we performed a double labelling of DMC mutants with an antibody raised against ubiquitin. As shown in Figure 8B, ubiquitin was found co-localized with mutant GFP-Dym aggregates. These results strongly suggest that DMC mutations result in intracellular deposition of mis-folded Dymeclin which then aggregates into ubiquitin-rich cytoplasmic inclusions, most likely inducing its degradation, whereas the E87K SMC mutation does not affect the stability and the location of the protein.

Discussion

Dymeclin, a new peripheral protein of the Golgi apparatus

The gene responsible for DMC syndrome encodes a 669-aminoacid protein we have named Dymeclin. BLASTS, sequence motif and homology searches using multiple databases indicated that Dymeclin is a novel evolutionarily conserved protein with no homology or known conserved functional domains, except for a putative N-myristoylation site (2). Using electron microscopy and immunofluorescence techniques on both fixed and living cells, we have shown in this study that Dymeclin is a peripheral protein of the Golgi apparatus. We did

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3 not observe any significant ER labeling, which is in contrast with data obtained by Osipovich
4 et al (13). However, these authors used an aminoterminally tagged Dymeclin, a tagging
5 position which hampers myristoylation of the protein. Despite many trials with either home-
6 made or commercially available antibodies (12 antibodies tested so far), specific signal for
7 endogenous Dymeclin was hardly detectable in our hands by Western blot. This may be due
8 to a poor ability of Dymeclin to elicit immune response or a high turn-over/instability of the
9 protein in solution. However, we could detect a Golgi/cytosolic signal for many of the anti-
10 Dym antibodies and immunoprecipitate Dym-GFP with one of them (see Fig. 2B). Based on
11 the hydrophobicity profile of the aminoacid sequence, Dymeclin was first thought to be an
12 integral membrane protein with six trans-membrane segments (1). Again, owing to the very
13 fast release of Dymeclin from the Golgi (Fig. 6) we could not get conclusive results from
14 classical biochemical fractionation. However, using imaging experiments, we have shown that
15 Dymeclin is not a stably anchored trans-membrane protein but a dynamically localized
16 peripheral protein of the Golgi apparatus. Firstly, short cell permeabilization without protein
17 extraction using digitonin resulted in a complete loss of both soluble and Golgi-localized
18 Dymeclin. In the same condition integral membrane proteins (not shown) but also well known
19 Golgi peripheral proteins like ARF1 and GRASP65 were still membrane-associated. In
20 addition, FRAP experiments revealed that Dymeclin shuttles very rapidly between the cytosol
21 and the Golgi apparatus. Interestingly, it is the fastest Golgi shuttling protein described so far,
22 exceeding the speed observed for ARF1 and GRASP65 (our results and (14, 15)). Dymeclin is
23 thus present as a cytosolic pool that can shuttle very rapidly between the cytosol and the Golgi
24 apparatus, like other myristoylated proteins. However, the Golgi localization of ARF1 or
25 GRASP65 depends on their myristoylation whereas Dymeclin as a second unknown
26 mechanism of localization. Another striking difference which we report here is that while
27 proteins like ARF1 or GRASP65 were not observed to select particular Golgi stacks,
28 Dymeclin dynamically recognizes a subset of the Golgi apparatus revealed by the
29 depolymerization of microtubules. The existence of two Golgi complex subsets was reported
30 previously (12) but only Giantin was found to preferentially localize on the “old”, pre-
31 existing, Golgi apparatus. That a shuttling protein like Dymeclin can similarly recognize the
32 “old” Golgi suggests that it can recognize Golgi components specifically found at the surface
33 of mature Golgi apparatus and not on newly formed mini-stacks (Fig. 9 B, green) in contrast
34 to other shuttling proteins like ARF1 (Fig. 9 B, red). Both to understand the role of Dymeclin
35 in disease development and to characterize further Golgi complex dynamics, it will be
36 important to understand how Dymeclin can recognize a subset of the Golgi apparatus and if

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3 specific proteins or lipids are necessary for this localization. Because Giantin is a
4 transmembrane protein of the Golgi apparatus, and is the only known marker of this mature
5 Golgi compartment, it is tempting to speculate that Dymeclin is recruited on Golgi
6 membranes by Giantin. Interestingly, Osipovich et al. identified Giantin as a potential
7 Dymeclin partner by mass spectrophotometry analysis although this result has not been
8 validated (13).
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13 ***DYM* mutations lead to complete degradation of Dymeclin in DMC but not in SMC.**

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16 Many mutations have been identified in DMC and SMC dysplasias which lie in various
17 locations along the *DYM* gene (3). Although most of them predict protein truncation and a
18 likely loss-of-function of Dymeclin, two different missense mutations have been associated
19 specifically with DMC syndrome (N469Y, (1 , 2)) or SMC syndrome (E87K, (1 , 2)). Since
20 mental retardation is constantly observed in DMC and absent in SMC, we asked whether
21 these two specific amino-acid substitutions could result in different consequences on
22 Dymeclin and found that the N469Y but not the E87K mutation results in mis-localization
23 and subsequent protein degradation. So far, *DYM* mutations associated with SMC syndrome
24 have been identified in only two families which share compound heterozygosity for the same
25 two mutations, namely the E87K substitution (on one allele) and a splicing mutation leading
26 to exon 8 skipping and a premature stop codon (on the other allele, (1 , 2)). Interestingly the
27 latter mutation has been associated with DMC as well (3). Since heterozygous individuals
28 who bear the exon 8 splice mutation plus one normal allele (like parents of these patients) are
29 not affected, the E87K mutation appears specific to SMC phenotype. Given that this particular
30 mutation neither results in Dymeclin degradation nor induces mis-localization, it is likely that
31 some residual activity of Dymeclin could explain the absence of neurological phenotype in
32 SMC patients. Conversely, since DMC and SMC share similar skeletal features, the E87K
33 mutation likely exerts a deleterious effect in bony tissue. It will be interesting to identify the
34 specific defects induced by the E87K mutation that prevent this Golgi-localized Dymeclin
35 form to normally fulfill its function.
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53 **What role for Dymeclin in DMC syndrome.**

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55 Although skin anomalies are not part of the DMC phenotype, Dymeclin is present in
56 fibroblasts and the ultrastructural anomalies observed in skin fibroblasts from DMC
57 individuals presumably properly reflect the intracellular consequences of *DYM* deficiency (3).
58 Indeed, electron microscopy analysis of chondrocytes from two children with Smith-McCort
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3 dysplasia (SMC) also revealed a highly dilated rough endoplasmic reticulum (6). From their
4 observation, the authors concluded that SMC and DMC should be rough endoplasmic
5 reticulum storage disorders. However, the specific compound which would accumulate does
6 not seem to be Dymeclin itself since both nonsense and missense DMC mutations result in
7 degradation of the protein. Several extensive biochemical analyses, including
8 incorporation/degradation assays of sulphated proteoglycans, peroxysomal and lysosomal
9 contents examination, lipid, carbohydrate and protein work up and assays of mitochondrial
10 respiratory chain activities were performed by our group and others. However, no enzymatic
11 deficiency or specific accumulated compound in DMC cells which would have been
12 consistent with a specific storage of a putative mis-degraded metabolite could be found (3 , 16
13 , 17 , 18 , 19). Although we can not rule out the possibility that DMC and SMC would be the
14 consequence of a metabolic perturbation which we were unable to characterize yet, our data
15 provide a body of arguments in favor of an ER-Golgi membrane trafficking disorder.
16 However, experiments we carried out on DMC fibroblasts failed to reveal a reproducible
17 defect in anterograde and retrograde membrane trafficking (data not shown). This may not be
18 surprising because fibroblast cells are to affected in DMC patients. This is nevertheless in
19 marked contrast with results reported by Osipovich et al., although the effects were not
20 quantified by the authors (13). Dymeclin has common features with other Golgi proteins such
21 as GRASP65 or ARF1. These three proteins are localized to the Golgi apparatus, they are
22 myristoylated and they shuttle rapidly between a cytosolic pool and a Golgi pool. Dymeclin
23 could therefore be involved in a GRASP like function and have a role in the structure and/or
24 the function of the Golgi apparatus. GRASP65 has been involved in the formation of the
25 mitotic spindle (20) but also in the fusion of the Golgi ribbons (21). In drosophila, the
26 GRASP65 homologue is involved in non-conventional secretion (22) A related protein,
27 GRASP55 was recently shown to be similarly involved in Golgi unlinking (23). Interestingly,
28 unlinked Golgi cisternae are reminiscent of nocodazole-generated mini-stacks that cannot be
29 recognized by Dymeclin. Structural defects were actually observed in patient cells which
30 further argue for a role of Dymeclin in the regulation of Golgi apparatus organization.
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32 Dymeclin could also have a role similar to the role played by ARF1 or Sar1 and be important
33 for the formation of a complex of proteins (like COPI or COPII respectively) involved in a
34 step of the intracellular transport (24 , 25). This hypothesis could also explain the defects
35 observed by electron microscopy in patient fibroblasts. In this context, it is interesting to note
36 that a mutation in a component of the COPII coat component Sec23A leads to cranio-
37 lenticulo-sutural dysplasia (26). In fibroblasts from these patients, the ER appears swollen and
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3 many tubular protrusions were observed (27). Sec23A is an ubiquitous protein, like Dymeclin,
4 which is essential for intracellular transport but only affects some cell types.
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6 Most of inherited diseases known to be associated with ER-Golgi anomalies involve cargo
7 molecules that become unable to couple to the export machinery and then either undergo
8 degradation or accumulate in the cell (28 , 29 , 30). So far, few genetic disorders have been
9 reported to involve central constituents of the ER-Golgi budding, presumably because their
10 disruption is lethal. One of them, however, the X-linked skeletal disorder Spondyloepiphyseal
11 dysplasia tarda (SEDT, MIM #313400), is viable and associated with a loss-of-function of
12 Sedlin, the functional counterpart of the yeast transport protein particle (TRAPP) Trs20p (31 ,
13 32). Interestingly, TRAPP is a large cytosolic protein complex (~ 1,000 kDa) required for
14 tethering ER-derived vesicles to Golgi membranes and for intra-Golgi traffic. In addition,
15 SEDT is characterized by several features strikingly reminiscent of DMC (3 , 33): i) both
16 diseases are chondrodysplasias with short-trunk dwarfism, ii) in both cases, the phenotype is
17 not obvious at birth and appears progressively in childhood and iii) in both diseases, clinical
18 features specifically include a broad chest with sternal protrusion and a flattened and humped
19 appearance of the vertebral bodies. Although Dymeclin does not share any sequence
20 homology with Sedlin, it is tempting to speculate that the two proteins may have a functional
21 relationship in cartilage differentiation.
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23 It will now be important to unravel Dymeclin's connection to other regulators of ER/Golgi
24 dynamics and function to understand better the role that this protein plays in the development
25 of the DMC/SMCdysplasias.
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35 **Materials and Methods**

36 **Multiple-tissue expression array and Northern blot**

37 Pre-made human multiple-tissue expression array (MTE; ref 7776-1 Clontech Laboratories)
38 and Northern blot containing 20 µg of total RNA extracted from human primary
39 chondrocytes, calvarial osteoblasts, immortalized chondrocytes, SaOs2, HeLa and murine ES
40 cells were hybridized overnight at 42°C with either a 0.85 kb cDNA fragment corresponding
41 to the 5'-end of *DYM* (probe A) or a 0.5 kb cDNA fragment selected in the 3'-end of *Dym*
42 (probe B), labelled and purified as previously described (2). Primers used to generate probe A
43 were selected in exons 2 and 9 (2). Primers used to generate probe B were selected in exon 10
44 (10Sintra: 5'-CTCCTCTTGATACCTTGCT-3') and exon 14 (8ASintra: 5'-
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3 AGGAACATCATTAGAACTCAG-3'). Blots were washed three times at 42 °C with
4 2×SSC : 0.1% SDS for 15 minutes and once under more stringent conditions, at 65 °C with
5 0.1×SSC : 0.1% SDS for 10 minutes and further exposed overnight to Kodak X-Omat films
6 with an intensifying screen at -80 °C.
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10 11 **In situ hybridization**

12 Human embryos and foetal tissues were collected from legally terminated pregnancies in
13 agreement with the French law and Ethics Committee recommendations. Tissues were fixed
14 in 4% paraformaldehyde, embedded in paraffin blocks and sectioned (5 µm). Primers located
15 in exon 7 (7F: 5'-AAGAAGTTTTGCGACAGAGC-3') and exon 9 (9R:5'-
16 GGCCAGAGGGGAAGAAAG-3') of *DYM* cDNA were selected for PCR amplification of a
17 315 bp product. A T7 promoter sequence extension (5'-
18 TAATACGACTCACTATAGGGAGA-3') was added at the 5' end of each primer. T7-7F/9R
19 and 7F/T7-9R sets allowed amplification of sense and antisense templates. Ribobropes were
20 labelled using T7 polymerase in the presence of α [³⁵S]UTP (1200 Ci/mmol; NEN) and
21 purified on Sephadex G50 columns. Hybridization and post-hybridization washes were
22 carried out according to standard protocols (34). Slides were dehydrated, exposed to
23 BIOMAX MR X-ray films (Amersham) for 3 days, dipped in Kodak NTB2 emulsion for 3
24 weeks at +4 °C, then developed and counterstained in toluidine blue, coverslipped with
25 Eukitt, and analysed under dark and bright field illumination. No hybridization signal was
26 detected with the α [³⁵S]-labelled sense probe.
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43 **Plasmids and site-directed mutagenesis**

44 The coding region of human *DYM* cDNA (FLJ90130, DDBJ/EMBL/Genbank accession
45 number AK074611) was purchased from the Biological Resource Center, Japan (NBRC) as
46 an insert cloned into the pME18SFL3 plasmid. However, the clone differed by two
47 nucleotides from the human sequence we previously identified (2) changing a glutamate into a
48 lysine at codon 66 and a leucine into a proline at codon 249. These two mismatches were
49 corrected using the QuickChange XL site directed *in vitro* mutagenesis kit, following the
50 manufacturer's instructions (Stratagene). The cDNA of *DYM* was amplified by PCR and
51 subcloned in the pEGFP-N2 vector (Clontech) between XhoI and BamHI. The QuickChange
52 XL site directed *in vitro* mutagenesis kit was used to generate the R204X, Q483X, K616X,
53 E87K and N469Y mutations. Primers designed to mutate the myristoylation site of Dymeclin
54 were as follow: G2Afor, 5'-AGCTTGAAGATGGCATCGAATAGCAGC-3', G2Arev, 5'-
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3 GCTGCTATTCGATGCCATCTTCAAGCT-3'. In FRAP analyses, we used pEGFP ARF1
4 and pEGFP GRASP65 (kindly provided by F. Barr). All constructs were verified by direct
5 sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit on an
6 automatic ABI3100 capillary sequencer (Applied Biosystems).
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10 11 **Cell culture and transfection**

12 HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing
13 4.5 g/l glucose supplemented with 10% fetal calf serum, 5 mM glutamine, 5 mM sodium
14 pyruvate at 37 °C in a 5% CO₂ humidified incubator. For transfections, cells were plated on
15 coverslips the day before transfection, and were transfected using lipofectamin 2000
16 (Invitrogen). The cells were fixed with either 100% methanol at -20°C for 5 minutes or 3%
17 PFA at room temperature for 15 minutes, washed with PBS, incubated with PBS-0,1M
18 NH₄Cl for 5 minutes, washed again with PBS and permeabilized with 0,1% Triton X100 for 5
19 minutes.
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30 **Immunoprecipitation**

31 Immunoprecipitations were carried out by using extracts prepared from HeLa cells over-
32 expressing Dym-GFP with the Protein G-agarose Immunoprecipitation kit from Roche.
33 Briefly, cell lysates were first incubated on a rocking platform 5 hours at 4 °C with 50 µl of
34 the homogeneous G-suspension and centrifuged 20 seconds at 12000 g. The supernatant was
35 then incubated with 2-4 µg of mouse monoclonal anti-GFP antibody (Roche) overnight at 4
36 °C and another 50 µl of the homogeneous G-suspension was added for 5 hours. After several
37 washes of the complexes, the immunoprecipitated proteins were separated by SDS-PAGE and
38 electroblotted on PVDF membranes (Immobilon, Millipore). The membranes were pre-
39 incubated for 1 hour at 4 °C in TBS (20 mM Tris-HCl pH7.4, 150 mM NaCl) containing 5%
40 skim milk. The blots were then incubated overnight at 4 °C with either anti-GFP (control) or
41 anti-Dym antibodies both at dilutions of 1/1000. After membrane washing, a second antibody
42 coupled to peroxidase was added. Proteins were visualized with the ECL detection kit
43 (Amersham).
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55 **Permeabilization assays and drugs**

56 For permeabilization assays, cells grown on a glass bottom chamber (Iwaki) were treated
57 directly during imaging with 60 µM digitonin for 1 minute. For Nocodazole treatment, cells
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3 were first incubated at 4 °C for 1h30, then at 37 °C for 1h30 in the presence of 10 μM
4 Nocodazole (Sigma) before being directly fixed or used for imaging.
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8 **Immunofluorescence**

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10 Primary antibodies: anti-giantin hFc TA10 1/50, polyclonal anti-GalT 1/1000, sheep TGN46
11 1/1000, anti-GM130 (Transduction Laboratories, 1/200), anti-transferin receptor OKT9 1/500,
12 DAPI 1/1000. Secondary antibodies: anti-human Cy3 (Jackson, 1/500), anti-rabbit Cy5 1/500,
13 anti-mouse Cy3 1/500, anti-sheep Cy3 1/500, anti-mouse Cy5 1/500, anti-human Cy5 1/500.
14 Fixed cells were visualized using a Leica DM6000B microscope with a CoolSnapHQ camera
15 (obj 100x NA 1.4 oil CS (HCX PL APO), or with a Leica DMRA and a Micromax 5 Mhz,
16 40x objective NA 1.25 oil ph3 CS (HCX PL APO), and Metamorph. 3D stacks were acquired
17 and deconvolved to build a projection on one plane on Metamorph.
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26 **Live imaging**

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28 For FRAP analyses with Dym-GFP, ARF1-GFP and GRASP65-GFP. HeLa cells were
29 maintained in culture medium in glass bottom chambers (Iwaki) and imaged on a Leica
30 DMIRE2 with a Roper Cascade II Camera, Polychrome V and a 100x NA 1.35 oil objective,
31 twenty-four hours after transfection. Images were collected before bleaching of the Golgi and
32 every 50 milliseconds after photobleaching. Images were processed using Metamorph
33 software. After correction of the photobleaching due to acquisition, the background was
34 subtracted. The intensity of fluorescence was then normalized and the half recovery was
35 quantified. For nocodazole treated cells the images were acquired on a spinning disk
36 microscope. The Spinning disk microscope is based on a CSU-22 Yokogawa head mounted
37 on an inverted TE-2000E Nikon microscope equipped with a motorized Märzhäuser XY
38 Stage. Images are acquired through a 60x 1.4NA Plan-Apo objective with a Photometrics
39 Coolsnap HQ2 CCD camera. Optical sectioning is achieved using a PI Pifoc piezo z-drive
40 mounted between the microscope turret and the objective. A Roper/Errol laser lounge is
41 equipped with 488 nm and 514 nm laser diodes, delivering 50 mW each, coupled to the
42 spinning disk head through a single fiber. Multi-dimensional acquisitions are performed in
43 streaming mode using Metamorph 7.1.7 software. Images were collected before bleaching of
44 the Golgi and every 400 milliseconds after photobleaching for Dym-GFP and mCherry-
45 GM130. The data shown in the movies were obtained using ND-SAFIR (N-Dimensional –
46 Structure Adaptive Filtering for Image Restoration) © INRIA/INRA 2007 as previously
47 described (35 , 36).
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Immunoelectron microscopy

Briefly, cells were fixed with 2% paraformaldehyde, 0.125% glutaraldehyde in culture medium for 30 minutes at 37 °C. This medium was removed and replaced by PFA 2% + 0,125% glutaraldehyde in phosphate buffer pH 7,4 for 2 hours at 37 °C. This fixative was replaced by PFA 2% in phosphate buffer pH 7,4. Cells were then processed for ultracryomicrotomy as previously described (37). Ultrathin cryosections were prepared with an ultracryomicrotome Ultracut FCS (Leica, Vienna, Austria) and immunogold labeled with the indicated primary antibodies and using protein A conjugated to 10 nm gold (Cell Microscopy Center, AZU, Utrecht, The Netherlands). Sections were analyzed under a Philips CM120 electron microscope, and digital acquisitions were made with a numeric camera Keen View (Soft Imaging Systems, Münster, Germany).

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Legends to figures

Figure 1: Expression of Dym throughout human development

A: Tissue distribution of human Dym transcripts. MTE array was probed with an 850 bp human Dym radiolabeled probe (probe A) as described in experimental procedures and spots corresponding to the most intense expressions (up to 100 arbitrary units) were quantified using Sigmagel (Jandel Scientific, v1.0). The identity of each cDNA dot is as follows: A1, whole brain, A2, cerebellum left, A3, substantia nigra, A4, heart, A5, esophagus, A6, colon transverse, A7, kidney, A8, lung, A9, liver, A10, leukaemia HL-60, A11, fetal brain, A12, yeast total RNA, B1, cerebral cortex, B2, cerebellum right, B3, nucleus accumbens, B4, aorta, B5, stomach, B6, colon desending, B7, skeletal muscle, B8, placenta, B9, pancreas, B10, HeLa S3, B11, fetal heart, B12, yeast tRNA, C1 frontal lobe, C2, corpus callosum, C3, thalamus, C4, atrium left, C5, duodenum, C6, rectum, C7, spleen, C8, bladder, C9, adrenal gland, C10, leukaemia K-562, C11, fetal kidney, C12, E.coli rRNA, D1, parietal lobe, D2, amygdala, D3, pituary gland, D4, atrium right, D5, jejunum, D7, thymus, D8, uterus, D9, thyroid gland, D10, leukaemia MOLT-4, D11, fetal liver, D12, E.coli DNA, E1, occipital lobe, E2, caudate nucleus, E3, spinal cord, E4, ventricle left, E5, ileum, E7, peripheral blood leukocytes, E8, prostate, E9, salivary gland, E10, Burkitt's lymphoma, Raji, E11, fetal spleen, E12, poly r(A), F1, temporal lobe, F2, hippocampus, F4, ventricle right, F5, ileocecum, F7, lymph node, F8, testis, F9, mammary gland, F10, Burkitt's lymphoma, Daudi, F11, fetal thymus, F12, human Cot-1 DNA, G1, paracentral gyrus of cerebral cortex, G2, medulla oblongata, G4, interventricular septum, G5, appendix, G7, bone marrow, G8, ovary, G10, colorectal adenocarcinoma SW480, G11, fetal lung, G12, human DNA 100ng, H1, pons, H2, putamen, H4, apex of the heart, H5, colon ascending, H7, trachea, H10, lung carcinoma, A549, H12, human DNA 500ng. No sample was spotted in F3-H3, D6-H6, H8, H9, H11 and G9. C, Northern blot analysis was performed using probe A, which recognizes two Dym variants (3.1 kb and 5.6 kb). The amount in each lane is normalized with RNA 18s.

B: In situ hybridization analysis of Dym transcripts during human embryo-foetal development. Hematoxylin/eosin stained sections under bright field illumination (A, D, F, H, J, L, N, P, R, T, V) and adjacent Dym-hybridized sections under dark field illumination (B, C, E, G, I, K, M, O, Q, S, U, W). A-C, Sagittal sections of a CS19 embryo (x10); as a negative control of hybridization, the sense Dym probe was used (C). D-E, Magnification of the lung

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3 (Lu, x25, D) and the intestinal epithelium (x32, F, G) corresponding to the black squares in A;
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5 White arrows point to elective signal seen in mesenchymal condensations (E). H-K,
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7 Transversal sections of the cerebellum from a 17-week foetus (x10, H, I) and x32
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9 magnification showing Dym expression in the external granule layer (J, K). L-M, Dym
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11 expression in the hippocampus from a 17-week foetus (x8). N-O, Dym expression in the
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13 neocortical plate and in the corresponding germinative zone from a 17-week foetus (x12). P-
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15 S, Dym expression in the cerebellum from a 22-week foetus (x8) and higher magnification
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17 showing Dym expression in the external granule layer, the Purkinje neurons and the internal
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19 granule layer (x40, R and S). T-U, Dym expression in the hippocampus from a 22-week
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21 foetus (x40). V-W, Dym expression in the neocortical plate and in the corresponding
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23 germinative zone from a 22-week foetus (x12).

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25 **Figure 2: Immuno-detection of endogeneous Dymeclin in HeLa cells. A:** Intracellular
26
27 localization of endogeneous Dymeclin in HeLa cells. HeLa cells (Top panel) were fixed and
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29 stained with the anti-Dym antibody at dilution 1/400. The Golgi was labeled with GM130
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31 (red). Hela cells transfected with Dym(N469Y)-GFP (Bottom panel) were fixed and stained
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33 for Dymeclin (red) and GM130 (pink). **B:** Immunoprecipitation of Dym-GFP. Lysates from
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35 HeLa cells over-expression Dym-GFP (lane 1) were immunoprecipitated with either the anti-
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37 GFP antibody (lane 2) or the anti-Dym antibody (lane 3).

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39 **Figure 3: Intracellular localization of Dym-GFP in HeLa cells. A:** Reconstruction from 3D
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41 images of HeLa cells transfected with Dym-GFP and stained with antibodies against Giantin
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43 (in red, top line) and GalT (in blue, top line) or TGN46 (in red, bottom line) and GM130 (in
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45 blue, bottom line). Dym-GFP partially co-localized with the different Golgi markers and was
46
47 also found as a soluble pool. **B:** Immunogold labeling on cryosections of Dym-GFP
48
49 transfected HeLa cells visualized by electron microscopy confirmed that the protein is
50
51 localized on the Golgi apparatus and in the cytosol.

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53 **Figure 4: Myristoylation of Dymeclin *in vitro* and intracellular localization of the G2A**
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55 **mutant in HeLa cells. A:** Dymeclin was produced *in vitro* in the presence of either ³⁵S-
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57 methionine (left panel) or ³H-myristate (right panel). Wild-type Dymeclin (Dym WT) can be
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59 myristoylated *in vitro* and the mutation of the second Glycine into an Alanine (Dym G2A)
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prevents the myristoylation. **B:** Hela cells were transfected with Dym-GFP or Dym-G2A-GFP
(green) and stained for GM130 (red).

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5 **Figure 5: *In vivo* permeabilization with Digitonin.** HeLa cells stably expressing mCherry-
6 Histone were transfected with either Dym-GFP, ARF1-GFP or GRASP65-GFP. 3D images
7 were acquired on a spinning disk microscope before treatment and one minute after treatment
8 with 60 μ M Digitonin. In permeabilized cells Dym-GFP has completely disappeared from the
9 Golgi and the cytosol whereas ARF1-GFP is dimly visible on the Golgi apparatus and
10 GRASP65-GFP is still present.
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17 **Figure 6: Quantification of Dym-GFP dynamics.** HeLa cells were transfected with either
18 Dym-GFP, Arf1-GFP or GRASP65-GFP and photobleaching experiments were performed 24
19 hours after. A. The Golgi apparatus of one cell (outlined in white in the movie) was bleached
20 after 10 seconds and images were then acquired every 100 mseconds for 1 minute (see mov.
21 1). After 13,5 seconds the fluorescence of Dym-GFP has totally recovered. From these
22 experiments we could quantify the Golgi fraction of the three proteins. The percentage of
23 protein on the Golgi is represented in B. 16,5 \pm 4% of Arf1-GFP, 13,6 \pm 3,9% of Dymeclin
24 and 33,2 \pm 10,8% of GRASP65-GFP are on the Golgi. The normalized intensity of
25 fluorescence for the three proteins was plotted on the same graph. The mean value is indicated
26 in black and the SD is indicated in grey. The half time of recovery was calculated from these
27 data as shown in C. GRASP65-GFP recovered more slowly with a half time of 12,4 \pm 4,5
28 seconds, the recovery of Arf1-GFP was faster with a half time of 7,1 \pm 2,6 seconds and Dym-
29 GFP was even faster with a half time of recovery of 2,8 \pm 0,9 seconds.
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43 **Figure 7: Intracellular localization and dynamics of Dym-GFP upon nocodazole**
44 **treatment. A:** HeLa cells transfected with Dym-GFP were fixed 24 hours after transfection
45 and the Golgi was labeled with GM130 (red) and Giantin (blue). In non treated cells (top line)
46 the three markers co-localized whereas after 1h30 at 4 $^{\circ}$ C and 1h30 at 37 $^{\circ}$ C with 10 μ M
47 nocodazole (bottom line) GM130 was present on the mini stacks dispersed in the cell and
48 Giantin and Dym-GFP were co-localized on the “Old Golgi” in the center of the cell. **B:**
49 FRAP experiments were performed on cells co-transfected with mCherry-GM130 and Dym-
50 GFP (see sup. Movie 2). The first line shows the Dym-GFP before (-1”), immediately after
51 (0”) or 9 seconds after (9”) the photobleaching (indicated by the white arrows). The second
52 line shows the mCherry-GM130 and the last line corresponds to the overlay of Dym-GFP in
53 green and mCherry-GM130 in red. The insert shows the upper bleach region with a three
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3 times magnification. Dym-GFP is present on the “Old Golgi” but not on the mini stacks that
4 are only positive for GM130. After photobleaching of the “Old Golgi” both Dym-GFP
5 fluorescence and mCherry-GM130 fluorescence are decreased. The recovery of Dym-GFP is
6 complete within 9 seconds whereas mCherry-GM130 has not recovered.
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12 **Figure 8: Mis-localization and degradation of Dymeclin bearing DMC mutations. A:**
13 HeLa cells transfected with wild-type (WT) or mutant GFP-Dym (K616X, Q483X, R204X,
14 N469Y, E87K) were fixed 24 hours after transfection and the Golgi was labeled with GM130
15 (red). **B:** HeLa cells transfected with GFP-Dym(R204X) or GFP-Dym(N469Y) were fixed 24
16 hours after transfection and labeled with an anti-ubiquitin antibody (red). Ubiquitin was found
17 co-localized with mutant GFP-Dym aggregates.
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25 **Figure 9: Schematic representation of Dymeclin dynamic localization. A:** This model
26 proposes that, in the non treated cells, Dymeclin (in green) and Arf1 (in red) are present as a
27 soluble pool and a Golgi pool and shuttle rapidly between the two (green and red arrows). **B:**
28 In nocodazole treated cells Arf1 is localized on the “Old Golgi” and on the newly formed
29 mini stacks whereas Dymeclin is present only on the “Old Golgi” and not on the mini stacks.
30 Both proteins exchange rapidly.
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39 **Movies captions (Quick Time format)**

40 **Movie 1: DymFRAP.mov**

41 FRAP analysis of Dym-GFP in live transfected HeLa cells showing the rapid replenishment
42 of Dym-GFP fluorescence in the Golgi apparatus after photobleaching. For details, see
43 Dimitrov et al, Figure 6.
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51 **Movie 2: Dym-GM130_Nz-FRAP.mov**

52 FRAP analysis of Dym-GFP and mCherry-GM130 in live transfected HeLa cells, incubated at
53 4°C for 1h30 and treated with 10µM nocodazole at 37°C, showing no replenishment of
54 mCherry-GM130 and rapid replenishment of Dym-GFP fluorescence in the “Old Golgi” after
55 photobleaching. For details, see Dimitrov et al, Figure 7.
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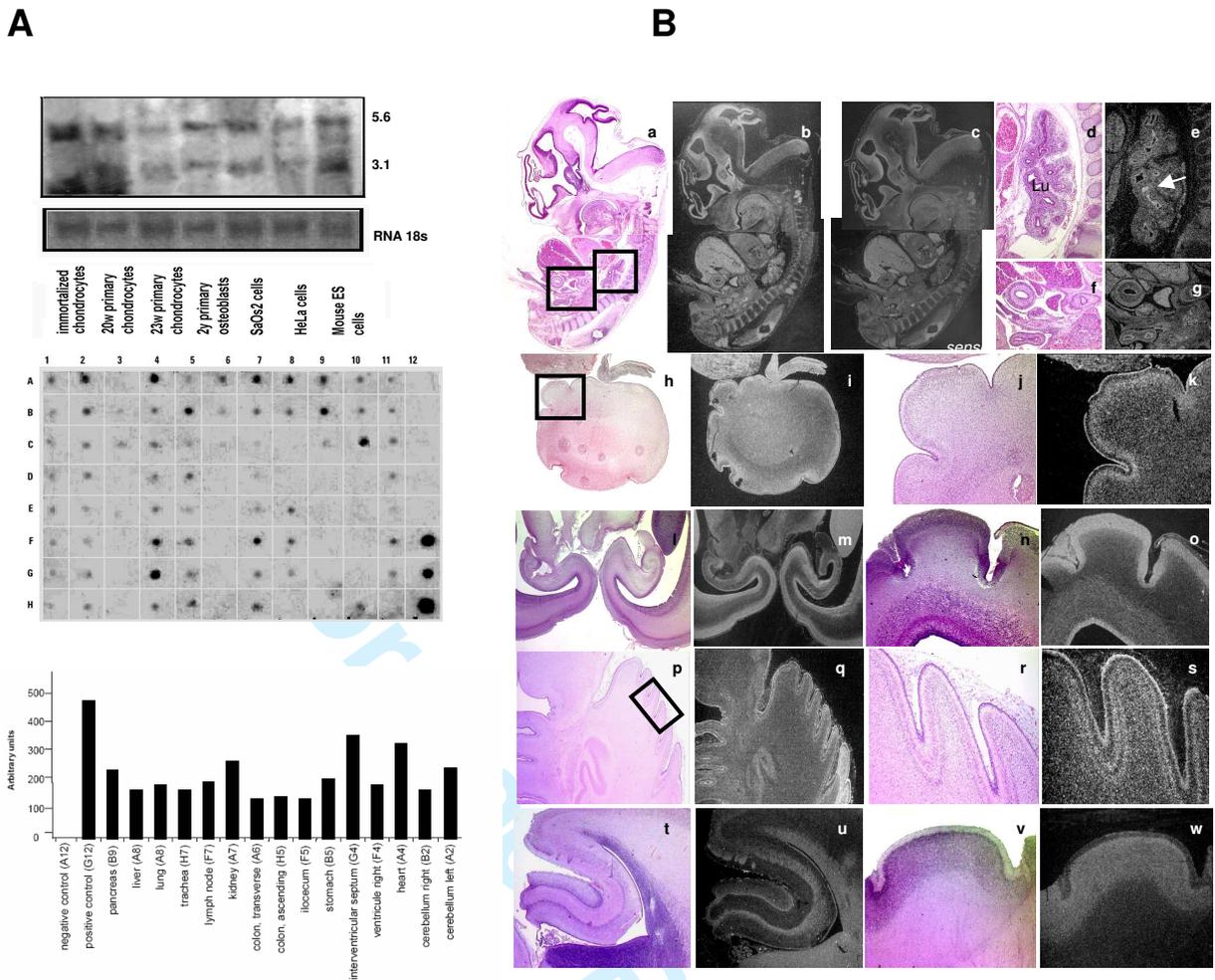


Figure 1, Dimitrov et al

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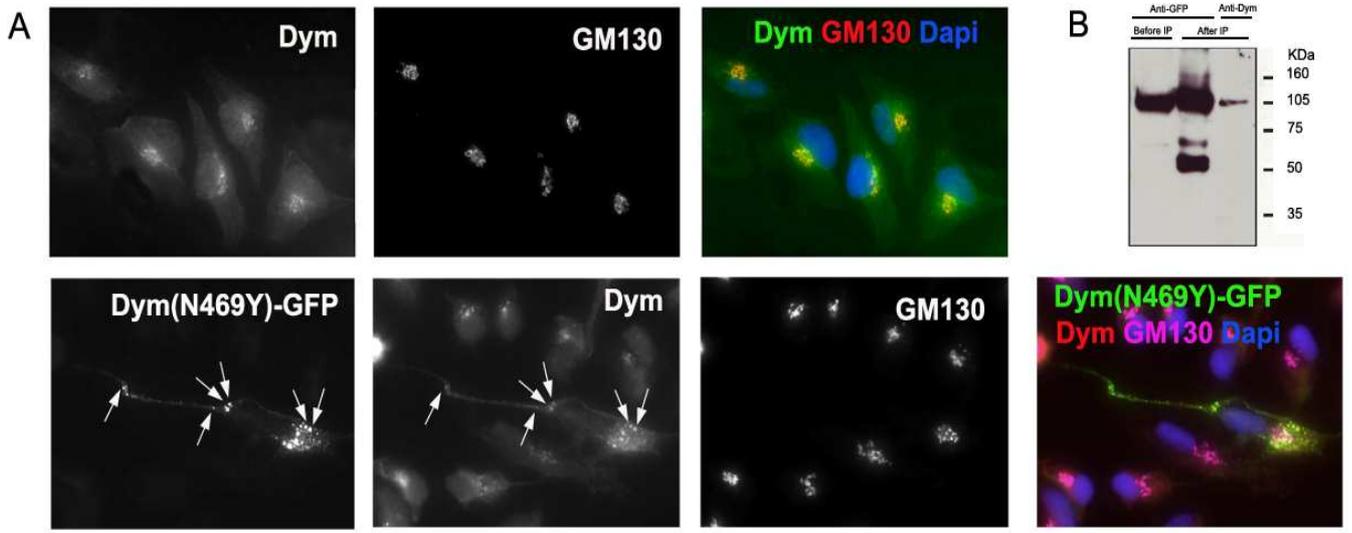


Figure 2, Dimitrov et al

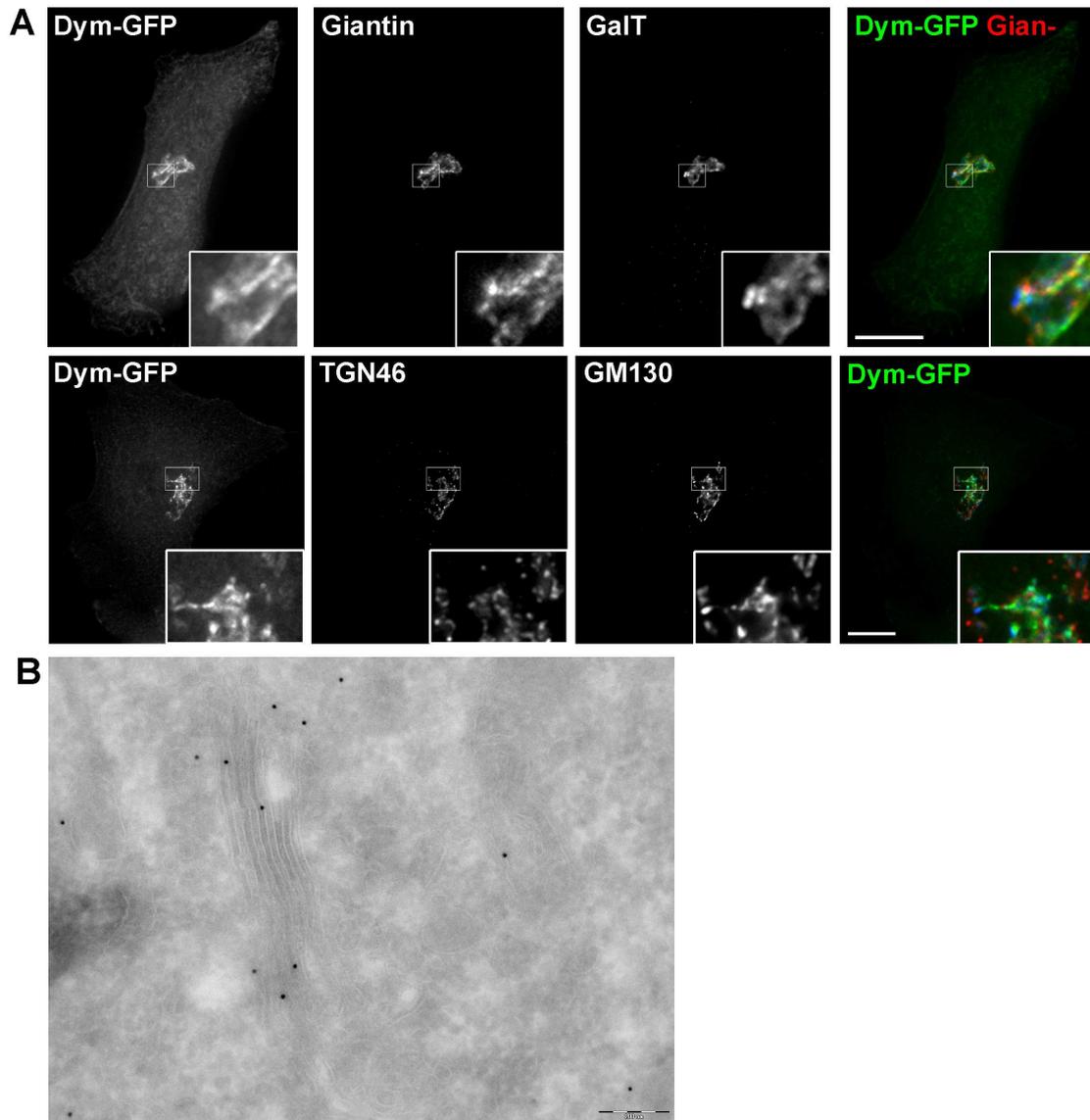


Figure 3, Dimitrov et al

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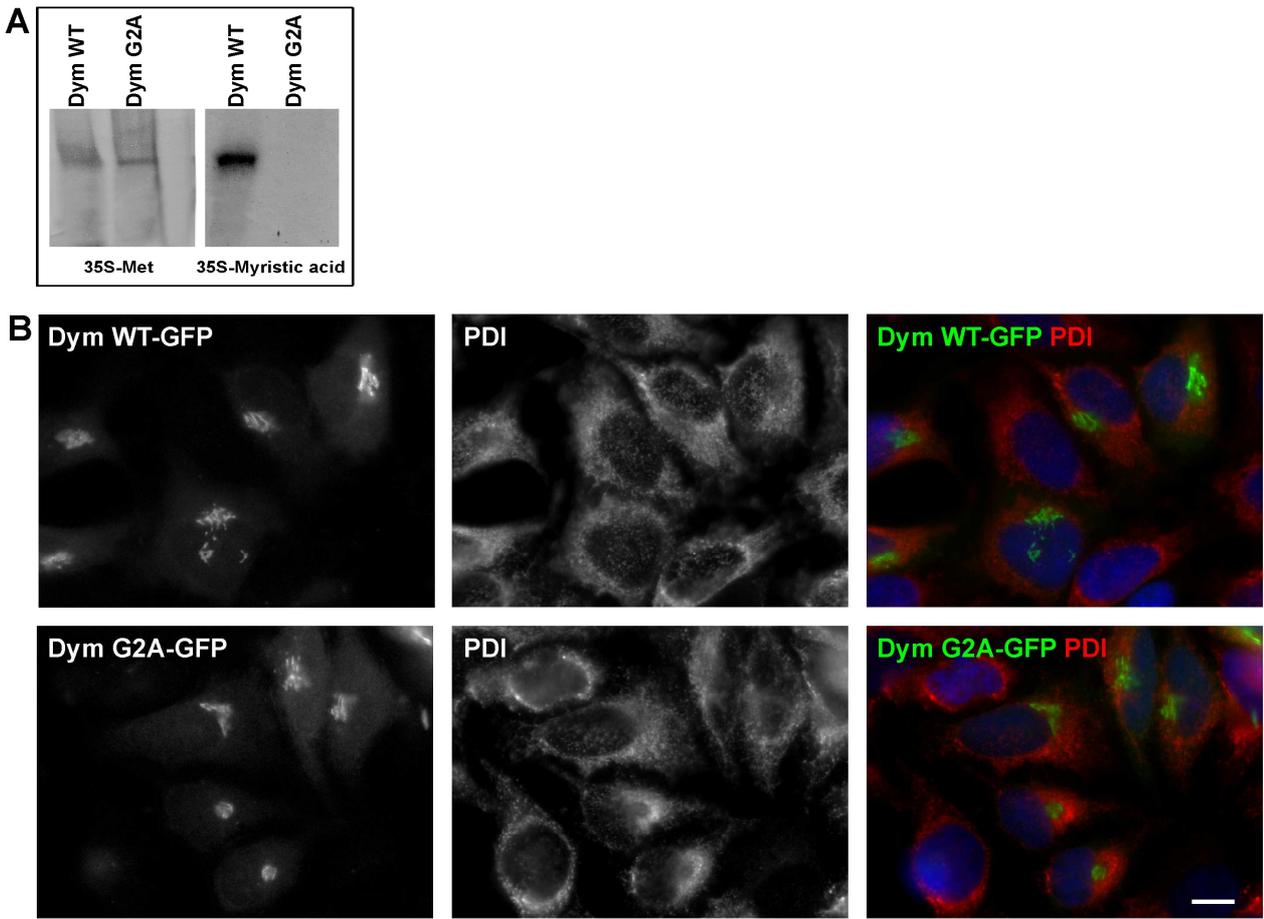


Figure 4, Dimitrov et al

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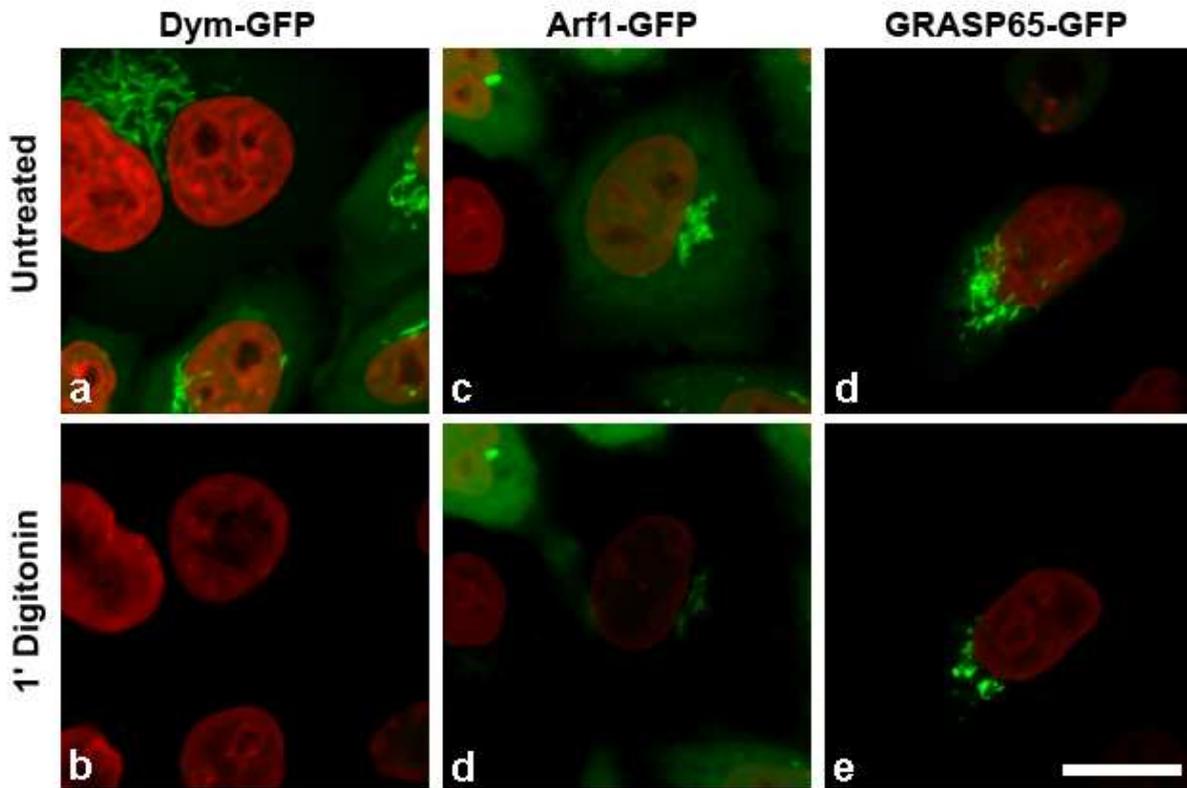


Figure 5, Dimitrov et al

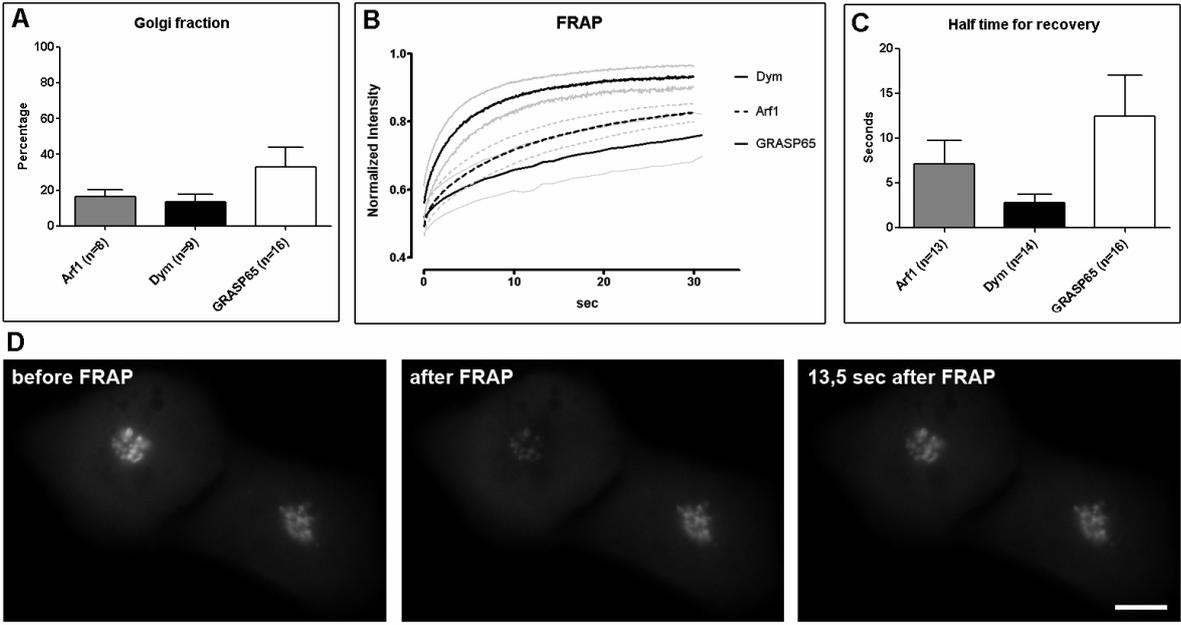


Figure 6, Dimitrov et al

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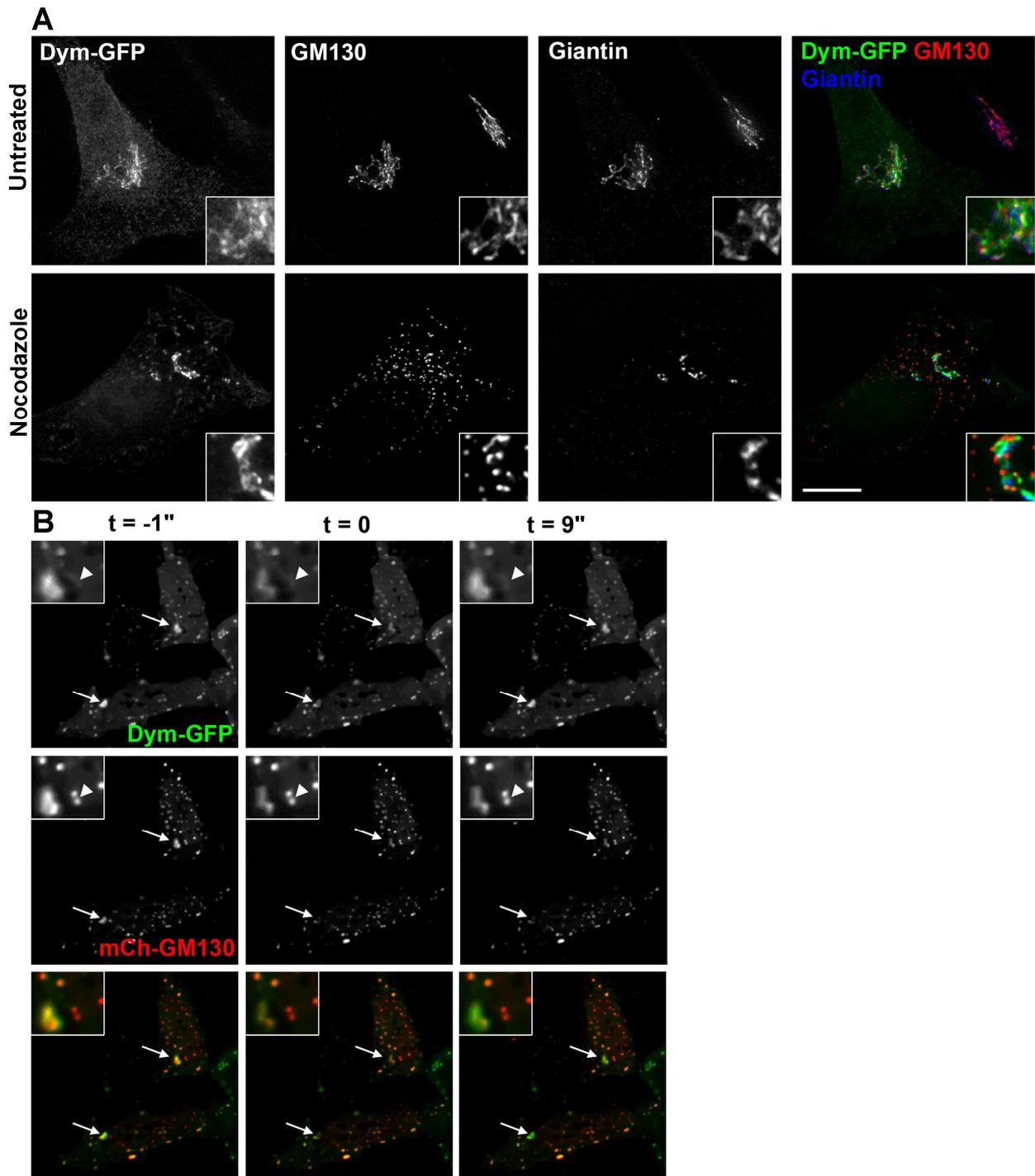


Figure 7, Dimitrov et al

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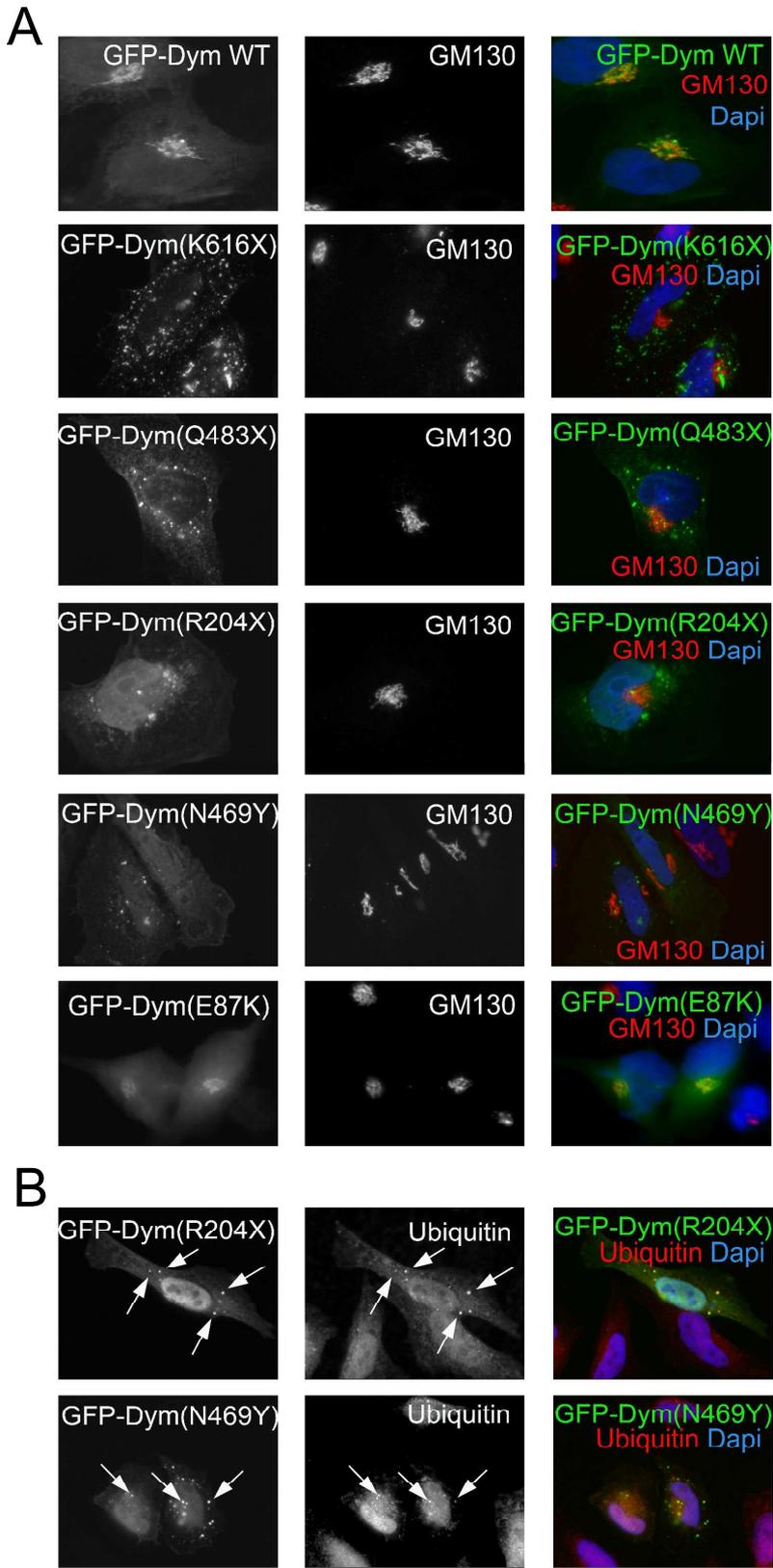


Figure 8, Dimitrov et al

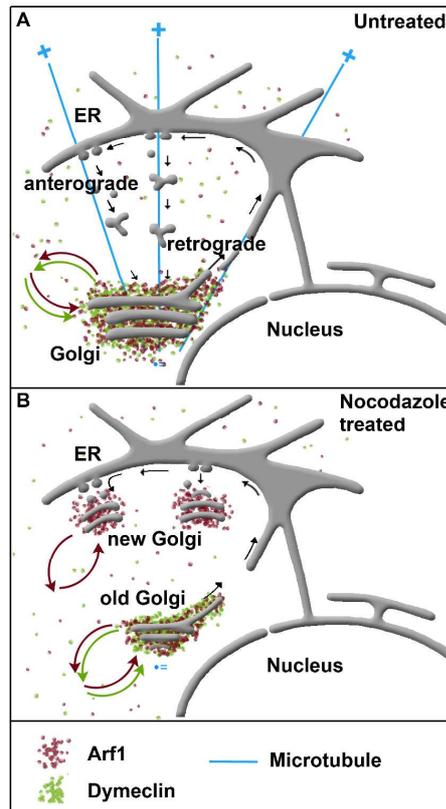


Figure 9, Dimitrov et al