**A simple and rapid immunoassay predicts dysferlinopathies in peripheral blood film**

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**List of abbreviations**

WB: Western blot
WBC: White blood cells
PBF: Peripheral blood film
IHC: Immunohistochemical
RT-PCR: Reverse transcription-polymerase chain reaction

**Declarations of interest:** none

**Abstract**

The assessment of dysferlin expression is useful to indicate or confirm the diagnosis of dysferlinopathies, a class of muscular diseases caused by mutations in the *DYSF* gene. Immunoblot analysis of skeletal muscle or monocytes is a specific and reliable diagnostic indicator of the disease, but the technique is specialized and laborious. We have developed a novel, robust immunoassay for detection of dysferlin in neutrophils requiring as little as one drop of blood. Our assay overcomes the issues of storage and handling of samples suggesting great promise as an inexpensive and rapid first screening for *DYSF* mutations. This relatively simple non-quantitative assay has the potential to benefit centers with limited resources, contributing to current diagnostic investigations into dysferlinopathies.

**Key words**

Muscular dystrophy, dysferlinopathy, immunohistochemistry, blood film

## 1. Introduction

Dysferlin is encoded by the *DYSF* gene on chromosome 2p13 as a protein of approximately 237 kDa. Dysferlin is widely expressed, but it predominates at the sarcolemma of striated muscle (1) where it is involved in membrane repair (2, 3), regeneration (4) and differentiation (5). Mutations in *DYSF* cause the autosomal recessive muscular disorders limb girdle muscular dystrophy R2 (LGMDR2 dysferlin-related, formerly LGMD2B, OMIM 253601), Miyoshi Myopathy (OMIM 254130), distal myopathy with anterior tibial onset (OMIM 606768) and congenital muscular dystrophy (1, 6-8). The large size of the *DYSF* gene has hindered a direct molecular diagnostic route. However, absence of dysferlin expression on Western blot (WB) is a highly specific diagnostic indicator of disease and it has become a widely used screening tool for dysferlinopathies (9, 10). Therefore, the diagnosis of dysferlinopathies requires a multidisciplinary approach that includes clinical evaluation and protein analysis ultimately confirmed by genetic analysis.

Peripheral blood is a mixture of platelets, erythrocytes and leucocytes or white blood cells (WBC) suspended in plasma. WBC are classified based on their cytoplasmic appearance

into agranulocytes (lymphocytes and monocytes) and granulocytes (neutrophils, eosinophils, and basophils) and are found in different relative proportions, with the neutrophils being the most abundant (60-70% of total WBC) (11). Ho et al (12) reported that dysferlin is expressed in $CD14^+$ peripheral blood monocytes ($CD14^+$) and developed a novel blood-based WB diagnostic assay. Although nowadays genetic tests are more accessible, the assessment of protein expression by WB of skeletal muscle or $CD14^+$ (12, 13) is still essential for validating the pathogenicity of unknown genetic variants and serves as an inclusion criteria for natural history studies (14). Blood-based assays such as $CD14^+$ WB and flow cytometry (15, 16) have gained popularity as being less expensive and invasive than muscle biopsy. $CD14^+$ represent only 3-7% of total leucocytes and selective enrichment of this cell population is required to yield an amount of sample suitable to the sensitivity of the tests. In addition, it is crucial that the blood sample is stored at 4°C and processed within a limited time frame to avoid protein degradation. Thus, these techniques have limitations: they are laborious, time consuming, require specialized equipment and expertise and their application is still restricted to specialized diagnostic laboratories.

Recent studies have demonstrated that dysferlin is also expressed in granules and lipid rafts of neutrophils (17-19). Here we demonstrate that detection of dysferlin expression on peripheral blood film (PBF) by enhanced immunohistochemical (IHC) methods is a suitable non-quantitative technique as a simplified, rapid and cost-effective diagnostic tool for dysferlinopathies.

## 2. Methods

### 2.1. Study participants and sample collection

All samples utilized in this study were kindly donated to the Newcastle MRC Centre for Rare Neuromuscular Disease Biobank (The Newcastle Hospitals NHS Foundation Trust, R&D Project ref: 5330) by healthy volunteers or patients of the Highly Specialized Service for LGMD at the Newcastle Hospitals NHS Foundation Trust. Informed consent was taken using Research Ethics Committee approved consent forms (REC reference: 08/HO906/28+5). Whole blood was collected directly via finger-pricks or drawn into EDTA vacutainers (Becton Dickinson). A total of 21 samples from genetically diagnosed dysferlinopathy patients, 2

asymptomatic carriers of dysferlinopathy, 13 healthy controls and 53 individuals affected by various neuromuscular diseases were assayed (Table 1).

### 2.2. Immunohistochemistry

All PBFs were prepared via the manual push method (20) and produced within 24 hours (h) of blood being drawn. Blood samples from 2 healthy controls were exposed to different storage temperatures (room temperature or 4°C) and time intervals between collection and processing over a 24h period (0h, 4h, 6h and 24h) and then used to produce PBFs. PBFs undergoing examination were left to air-dry for 30 minutes unfixed. Slides were labelled immediately or stored up to 2 years at room temperature until use. Air dried PBFs were fixed in methanol for 5 minutes and air dried for 1 minute before 15 minutes permeabilization in TBS (0.1% Triton X-100 (Sigma) in PBS pH7.3). Samples were then incubated for 2h with the anti-dysferlin antibody (Leica; NCL- Hamlet) followed by 3x5 minutes washes in TBS. X-Cell Plus Universal probe was then applied, followed by X-Cell Plus Polymer HRP and developed with Liquid Stable DAB (A. Menarini Diagnostics) according with the manufacturer's instructions. Nuclei were counterstained with Carazzi's hematoxylin.

### 2.3. Isolation of Neutrophil population

Whole blood samples from 7 dysferlinopathy and 3 healthy control donors were drawn into anticoagulant EDTA vacutainers and processed at varying time points for further analysis. Neutrophil populations were separated either immediately (0h), after 1h or following overnight incubation at room temperature. Neutrophils were isolated using the MACSxpress® Human Neutrophil Isolation Kit (Miltenyi Biotec Ltd.) according to manufacturer's instructions. Neutrophil fraction was then centrifuged at 500g for 5 minutes, supernatant removed and cell pellets frozen at -140°C for Western blot.

### 2.4. Western blot analysis

Neutrophil cell pellets (average $7x10^6$ cells) were thawed at 37°C and were resuspended and washed in PBS pH7.3 in order to remove any residual reagent from the isolation kit, then centrifuged at 10,000g for 2 minutes. The cell pellets were homogenized in 150 µl of treatment buffer containing 0.125 mol/L Tris/HCl buffer, pH6.8, 10% glycerol,

4% SDS, 4 mol/L urea, 10% mercaptoethanol, and 0.001% bromophenol blue. 70 µl were loaded in each lane. Western blots were conducted as previously described (9). Nitrocellulose membranes were incubated in NCL-Hamlet antibody and subsequently in HRP-conjugated rabbit anti-mouse antibody (Agilent) at room temperature. Bands were detected with Supersignal West Pico Chemiluminescent substrate (ThermoFisher Scientific) and acquired with FluorChem™ Q imager. Molecular mass was estimated with AlphaView software. Densitometry on archived diagnostic WB was performed with ImageJ software (NIH).

### 2.5. Neutrophil RNA analysis

Total cellular RNA was isolated from 2 healthy control neutrophil and 1 myoblast cell pellets using the QIAamp RNA Blood Mini Kit (Qiagen) following manufacturer's protocol. Reverse transcription was performed using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) with approximately 1µg total RNA as starting template. *DYSF* sequences were PCR-amplified using HotStarTaq DNA Polymerase (Qiagen) using specific *DYSF* primers (available upon request) with *GAPDH* primers used as an endogenous control. PCR products were ran on 1% agarose gels and detected by SafeView staining (NBS Biologicals). Product bands were excised from the agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen) and Sanger sequenced (Eurofins Genomics).

## 3. Results

### 3.1. Detection of Dysferlin Expression in PBF

Dysferlin labelling of PBF from healthy individuals showed expression on monocyte cells surface (Fig. 1A). Perinuclear and cytoplasmic labelling was also clearly seen in neutrophils. Dysferlin did not appear to be expressed in lymphocytes, while a faint cytoplasmic background coloration was detected in eosinophils (Fig. 1A). Identical results were obtained from venous and capillary blood. Given the relatively brief neutrophil lifespan, we assessed the kinetics of dysferlin persistence in controls up to 24 hours after blood sampling. Although the morphology of leucocytes was variably affected, particularly

in samples stored at room temperature, cytoplasmic labelling was retained at all time points analyzed. (Fig. 1B).

### 3.2. Dysferlin is expressed as multiple bands in neutrophils

To further characterize dysferlin expression in neutrophils, we undertook WB analysis on a pure cell population. In controls, dysferlin was generally detected as multiple bands (Fig. 2A). The uppermost band was of slightly lower molecular mass compared to muscle dysferlin and apparently of similar size (~220 kDa) as the lower band of the doublet described in $CD14^+$ (13). We observed a very faint band of expected size for the full-length protein only in two instances (not shown), but the result was not reproducible in samples obtained either from the same or other control individuals. Otherwise an identical pattern of bands of lower molecular mass was observed regardless of the time elapsed between blood draw and processing. The specificity of the labelling was demonstrated by absence of all bands in neutrophils from 7 dysferlinopathy patients (Fig. 2A).

### 3.3. Dysferlin RNA is alternatively spliced in neutrophils

To validate the expression of dysferlin in neutrophils, we performed reverse transcription-polymerase chain reaction (RT-PCR) analysis on isolated control cells with primers including regions previously reported as being alternatively spliced in blood and muscle dysferlin transcripts (21). Dysferlin expression was observed in both neutrophils and myoblasts. Alternative splicing patterns were confirmed in neutrophils, with inclusion of exon 5a and 40a and exclusion of exon 17 (Fig. 2B). No novel splicing events were observed within the range of the primers used. Alternatively spliced transcripts were Sanger sequenced with results affirming the observed splicing patterns.

### 3.4. Dysferlin absence in neutrophils is a hallmark of dysferlinopathies

In order to investigate whether our IHC protocol would be suitable to assess dysferlin expression in a diagnostic context, we carried out the test on blood samples collected from 21 patients with genetic diagnosis of dysferlinopathy. Protein expression in muscle biopsy had been previously assessed by WB in 13 of the patients in our cohort and reported as negative or extremely reduced by our diagnostic laboratory. Densitometric analysis of archived WBs showed residual protein expression ≤20% in all patients with one

exception (DYSF2, Fig. 3), where the intensity of the dysferlin band was ~23% compared with control. Only in this patient we detected a clear reduction of dysferlin labelling on PBF, which was otherwise absent from all the other dysferlinopathy samples analyzed. Any reduction of signal in the two dysferlinopathy carriers analyzed (DYSFC, Fig. 3) was not clearly detectable or quantifiable. In order to verify whether our results specifically indicate defects in the *DYSF* gene, we tested a cohort of 43 donors with various diagnosis of neuromuscular disorders and 11 patients with undiagnosed myopathies (Table 1). Presence of dysferlin labelling was observed in all disease controls regardless of diagnosis, age or gender (Fig. 3 and not shown). Slides from 13 controls, 16 dysferlin patients and 14 disease controls stored at room temperature for two years were labelled with no effect on the outcome of the test.

**4. Discussion**

Although the presence of dysferlin in neutrophils had been previously described (16, 17, 19), to date its expression had not been investigated in the diagnostic context. The specific role dysferlin plays in neutrophil function remains unknown. Work by Jethwaney et al. demonstrated that secretagogue treatment elicits the translocation of dysferlin from secretory to plasma membrane vesicles, suggesting that in neutrophils dysferlin mediates the fusion of secretory vesicles with plasma membrane in response to proinflammatory stimuli (19). In addition, dysferlin deficiency in monocytes has been shown to enhance phagocytotic activity, contributing to an overaggressive immune cell response (22). Questions remain unanswered as to whether dysferlin could also alter the phagocytotic activity of neutrophils. Further characterization of the function of dysferlin in WBC may provide important insights into the pathogenesis of dysferlinopathies and shed light on the etiology of the extensive muscle inflammation observed in dysferlinopathy patients.

Our assay aims to fulfil the need for a quick pre-screening to detect absence of dysferlin if a muscle biopsy is unavailable or the laboratory does not have facilities for $CD14^+$ isolation and immunoblot. Dysferlin expression in all but one patients in our cohort was either present or absent. Residual amount of dysferlin in dysferlin patient blood has been estimated as ≤20% compared to controls, with the majority of the patients showing ≤10% of

expression (12, 13, 15, 23). Therefore, the ability to appreciate reduction of labelling in blood from a patient with ~23% of residual dysferlin expression in skeletal muscle is promising in regard to the sensitivity of our test. Analysis of a larger cohort of patients is in progress and will help us gather more information on the threshold of sensitivity of the assay in cases where there is residual expression of dysferlin. Further protein quantification by WB should always be performed when the reduction in dysferlin labelling is unclear and a diagnosis must be confirmed by DNA analysis.

The non-quantitative nature of our test does not make it suitable to replace immunoblot assays in the assessment of residual expression of dysferlin. Our data, albeit limited, indicate that the identification of carrier status by IHC would be challenging. Also the presence of multiple specific bands on neutrophils WB would not allow the use of this technique for dysferlin quantification. Neutrophils activate under mechanical stress and even a minor strain can alter their state (24). The very rare detection of traces of full-length protein indicate that dysferlin may be degraded very rapidly during cell manipulation. The observation of an identical pattern of bands in samples processed at different time points suggests that protein degradation occurs very rapidly and does not progress over time. In addition, tissue-specific dysferlin isoforms may be expressed in neutrophils, as suggested by the presence of smaller transcripts of ~4kb and 2.0kb detected in total RNA isolated from blood (12).

Detection of dysferlin in neutrophils will aid the current diagnostic protocols as these cells are largely more abundant than monocytes and are readily recognizable in a standard PBF. This newly developed technique is non-invasive and requires as little as one drop of blood. Further processing of the sample is not required and a diagnostic outcome can be obtained within a few hours from blood drawing. In addition, detection of dysferlin on PBF overcomes the hurdle of storage and specialized handling of muscle and $CD14^+$ specimens since slides can be stored at room temperature for up to two years with no consequence on the accuracy of the test. This technique represents a less costly alternative to WB of either muscle biopsy or $CD14^+$ and requires only the basic equipment available in any histopathology laboratory. The result is visually intuitive and easily interpretable to detect presence, absence or extreme reduction of dysferlin.

The neuromuscular field is quickly moving toward trial readiness and a clearer understanding of genetics and prevalence of dysferlinopathies is essential. The current estimate of 7.5/million prevalence (25) is based on the incidence of known pathogenic mutations and doesn't include variants of unknown significance. Therefore, the true prevalence of the disease is still unclear and likely higher than estimated. Our method fulfils the need of a simple assay to evaluate variant pathogenicity leading to loss of dysferlin expression.

## 5. Conclusion

Our assay provides a cheaper, faster and simpler method to assess dysferlin expression. The ease of sampling makes it also suitable to neonatal and large population screening and its simplicity allows application even in centers with limited resources.


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**Figure legends**

**Figure 1. A: Dysferlin immunolabelling of PBF.** Dysferlin expression was detected in control monocytes (M) and neutrophils (N). The antibody did not specifically react with lymphocytes (L) and eosinophils (E). No labelling in neutrophils (N) was detected when the primary antibody was omitted (No Ab). **B: Retention of dysferlin labelling in neutrophils cytoplasm.** Dysferlin labelling of PBF from control blood samples stored at room temperature (right column) or 4°C (left column). Samples were smeared at varying time points (0h, 2h, 4h, 6h and 24h). Scale bars 25 µm.

**Figure 2. A: Western blot analysis of isolated neutrophils.** Left: Dysferlin is detected as multiple bands in control samples processed at different time points after blood drawing (C T0, 1H and OverNight). The estimated molecular mass of bands detected is indicated on the right. No bands are detected in a dysferlinopathy sample (DYSF T0). C SK: control skeletal muscle. Right: Ponceau S staining shows comparable amount of protein loading in neutrophils samples. **B: RT-PCR analysis of Dysferlin expression.** Dysferlin expression in neutrophils (N) and myoblasts (M) was confirmed via RT-PCR. Alternate splicing was observed also across exons 3-7, 14-20 and 38-41. Upper bands corresponded to inclusion and lower bands exclusion of exons 5a, 17 and 40a in dysferlin transcripts. Endogenous GAPDH control not shown.

**Figure 3. Comparative IHC of neutrophil dysferlin.** Representative results of dysferlin IHC on neutrophils from control (C), asymptomatic carrier of dysferlinopathy (DYSFC), dysferlinopathy (DYSF1, DYSF2), Bethlem Myopathy (BM) and facioscapulohumeral muscular dystrophy (FSHD) patients. Scale bar 10 µm. WB analysis of muscle samples shows complete absence of dysferlin in DYSF1 and a very clear reduction in DYSF2 dysferlinopathy patients. C-terminal dystrophin band is shown as loading control (DYS-C).

**Table 1:** Summary diagnostics of the study donors. Former LGMD nomenclature is indicated in parenthesis.

| *Disease* | *Disease Acronym* | *N* | *Sex* | *Median Age (range)* |
|---|---|---|---|---|
| *Primary Disease Cohort* | | | | |
| Dysferlinopathy | LGMDR2 (LGMD2B) | 21 | M/F | 40.3 (19-73) |
| *Primary Disease Carrier Cohort* | | | | |
| Dysferlinopathy asymptomatic carrier | | 2 | M/F | 48.5 (45-52) |
| *Healthy Control Cohort* | | | | |
| Healthy Control | | 13 | M/F | 38.6 (24-59) |
| *Disease Control Cohort* | | | | |
| Genetically undiagnosed myopathy | | 11 | M/F | 46.1 (20-65) |
| Facioscapulohumeral Muscular Dystrophy | FSHD | 8 | M/F | 51.9 (37-69) |
| Calpainopathy | LGMDR1 (LGMD2A) | 8 | M/F | 40.7 (22-57) |
| Becker Muscular Dystrophy | BMD | 5 | M | 44.2 (30-62) |
| Bethlem Myopathy | BM | 3 | M/F | 39 (31-48) |
| Spinal Muscular Atrophy II | SMA II | 2 | M/F | 30.5 (24-37) |
| Duchenne Muscular Dystrophy | DMD | 2 | M | 22 (21-23) |
| Anoctaminopathy | LGMDR12 (LGMD2L) | 2 | M | 57 (51-63) |
| Laminopathy | EDMD (LGMD1B) | 1 | M | 67 |
| Desminopathy | MFM1 (LGMD1E) | 1 | F | 61 |
| Dystroglycanopathy | LGMDR9 (LGMD2I) | 1 | M | 38 |
| Limb Girdle Muscular Dystrophy TRIM32 | LGMDR8 (LGMD2H) | 1 | F | 54 |
| Congenital Myasthenic Syndrome | CMS | 1 | M | 19 |
| Facioscapulohumeral Muscular Dystrophy Type 2 | FSHD2 | 1 | F | 66 |
| Nemaline Myopathy | NEM1 | 1 | M | 49 |
| Hereditary myopathy with early respiratory failure | HMERF | 1 | M | 56 |
| RYR1 Congenital Muscular Dystrophy | RYR1 | 1 | M | 64 |
| Inclusion body myopathy with Paget disease and frontotemporal dementia | IBMPFD | 1 | F | 61 |
| Welander Distal Myopathy | WDM | 1 | F | 75 |
| Congenital Muscular Dystrophy Type 1A | MDC1A | 1 | F | 38 |

Figure 1

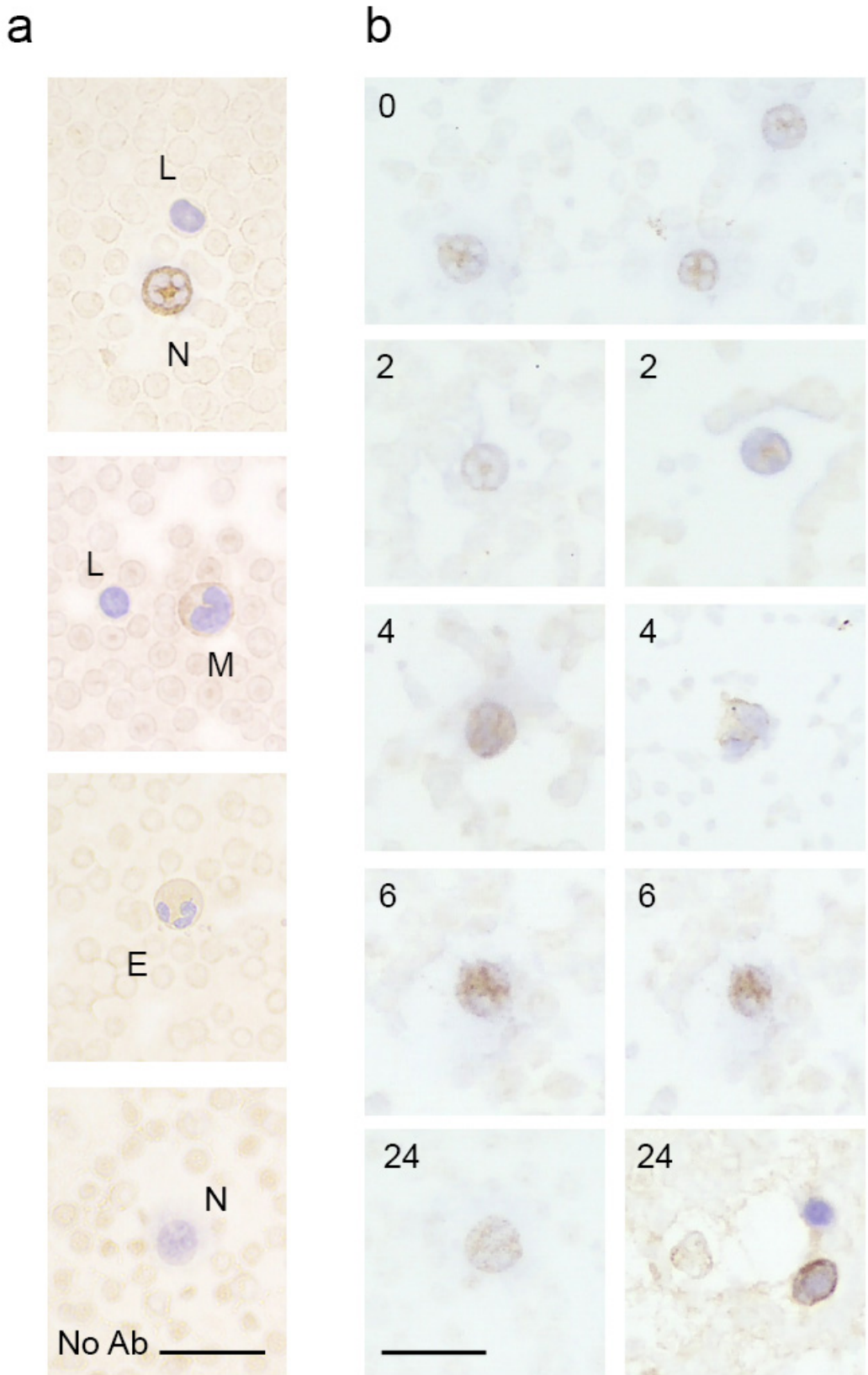

Figure 2

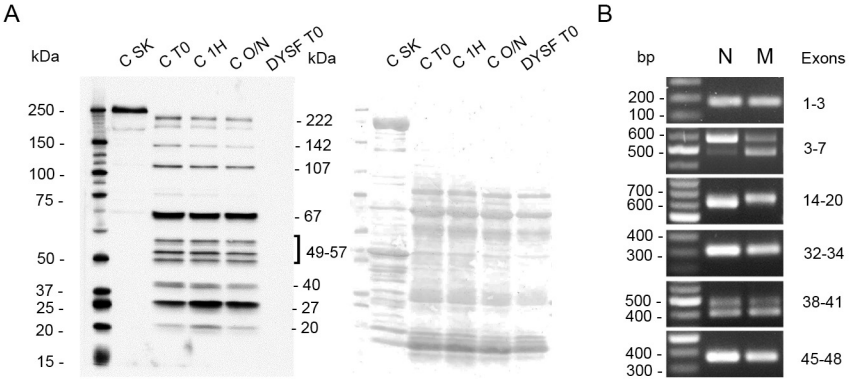
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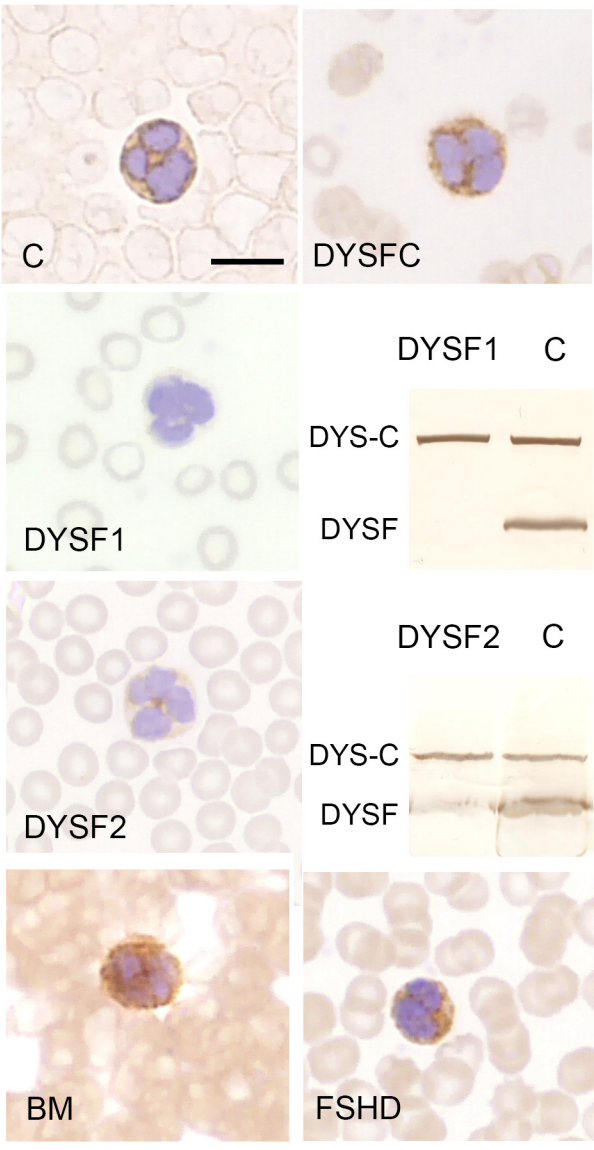
Figure 3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