Update on functional studies of YBX3 variants associated with nemaline myopathy

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INTRO

The Y-box binding protein 3 (*YBX3*) gene is highly expressed in skeletal and heart muscle. In the differentiation of skeletal muscle, YBX3 dephosphorylates and accumulates in the nucleus, simultaneously repressing myogenin transcription[1]. The *YBX3* missense variants studied here (p.S34R and p.R129W) were found by exome sequencing in a Finnish patient with an unusual form of nemaline myopathy (**Figure 1**).

The variants lie in the DNA-binding domain of YBX3[2]. The Ser34 has been shown to be phosphorylated and has been implicated as an important factor for nuclear YBX3 complex formation and its binding to single stranded DNA. The Arg129 is close to Ser134, which in turn is an implicated phosphorylation/dephosphorylation site relevant to the nuclear/cytoplasmic trafficking of YBX3.

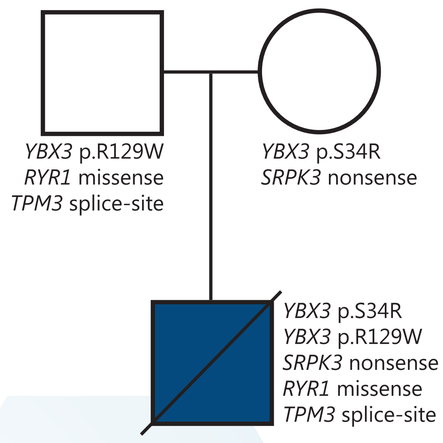

Figure 1. Pedigree of the Finnish nemaline myopathy patient. The patient has inherited one YBX3 variant from each parent. In addition, the patient has inherited a *SRPK3* nonsense variant from his mother, and the father and the patient also carry seemingly recessive variants in *RYR1* and *TPM3*.

RESULTS

R129W AFFECTS THE LOCALIZATION OF YBX3

Immunofluorescence imaging suggested, that the R129W variant causes YBX3 to localise in the nucleus more than the wild-type or the S34R variant (**Figure 2**). The localisation was quantified by CellInsight High Content Imaging analysis (HCI, **Figure 3**), confirming the result.

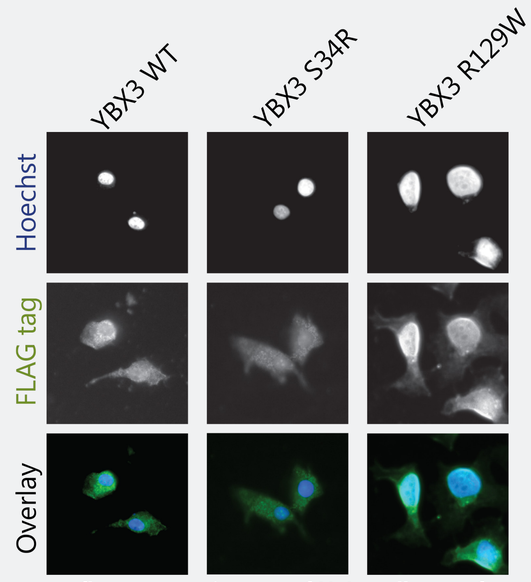

Figure 2. Immunofluorescence images of HeLa cells transfected with C-terminally FLAG-tagged YBX3 constructs stained with FLAG-tag recognising antibodies and Hoechst.

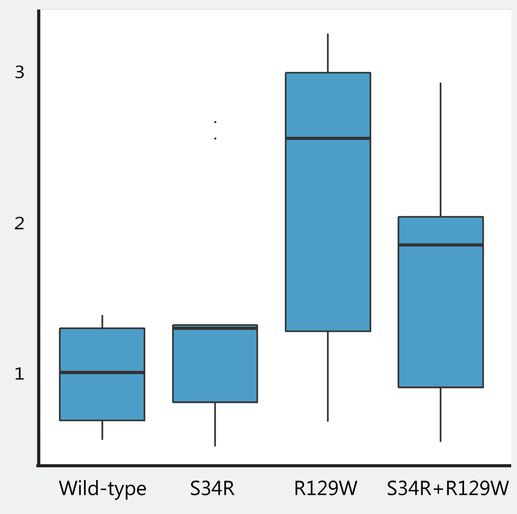

Figure 3. Relative intensity difference between the nucleus and the cytoplasm in HeLa cells transfected with C-terminally FLAG-tagged YBX3 constructs. All values were normalised to the wild-type average value. No significant difference was found between the wild-type and S34R. A significant difference was found between the wild-type and the R129W and the cotransfected cells ($p<0.01$ and $p<0.05$ respectively).

YBX3 IS CLEAVED & LOSES SOLUBILITY WHEN CLEAVED, ONLY THE END REMAINS

Western blots of soluble and insoluble fractions of whole cell extracts showed bands corresponding to the full length protein. In addition, a previously unrecorded shorter C-terminal peptide (**Figure 4**). To determine the fate of the N-terminal post-cleaving, Western blots of whole-cell extracts of cells transfected with both C- and N-terminally tagged constructs were made. The blots revealed that the N-terminal is absent in its cleaved state (**Figure 5**). Furthermore, the effect of the S34R variant on post-translational modifications is suggested in both blots by its seemingly slightly smaller size.

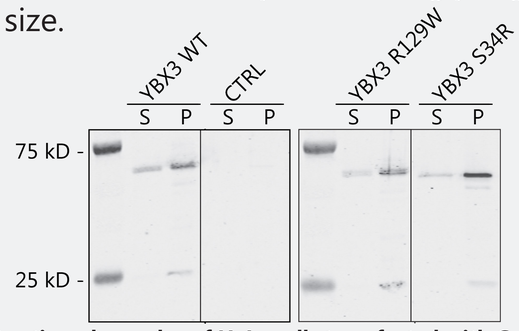

Figure 4. Fractioned samples of HeLa cells transfected with C-terminally FLAG-tagged YBX3-constructs, immunoblotted with a FLAG-tag recognising antibody. (S=soluble, P=pellet)

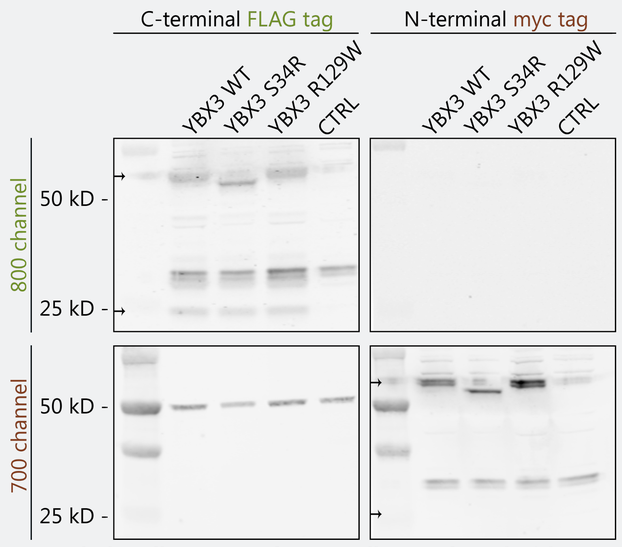

Figure 5. Whole cell extracts of COS1 cells transfected with N-terminally myc-tagged and C-terminally FLAG-tagged YBX3 constructs, immunoblotted with FLAG- and myc-tag recognising antibodies. β-tubulin was used as a loading control (FLAG-tag stain, 700 channel). The blot also demonstrates the effect of the S34R variant on the protein's post-translational modifications, as it travels faster through the blot.

THE C-TERMINAL LOCALISES INDEPENDENTLY

Immunofluorescence imaging of COS1 cells transfected with both C- and N-terminally tagged constructs (**Figure 6**) demonstrated, that the significant increase in nuclear localisation in R129W transfected cells (**Figure 3**) is dependent on the localisation of the C-terminal peptide fragment. The myc stain shows a perinuclear accumulation of the whole length protein[3]. CellInsight HCI analysis confirmed, that the C-terminal nuclear localisation is significantly increased as compared to the N-terminal (**Figure 7**).

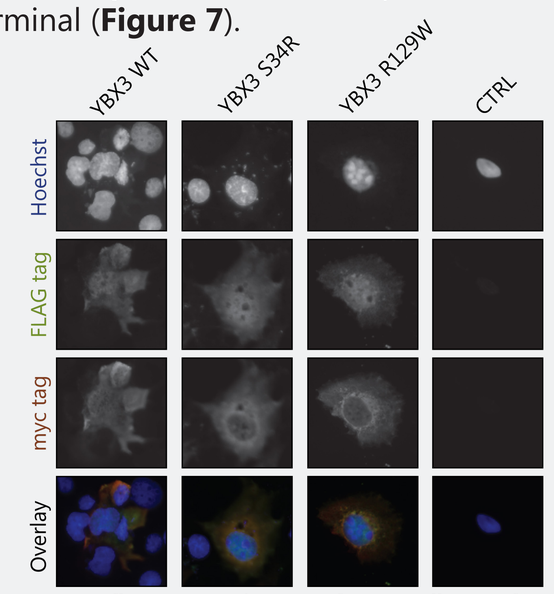

Figure 6. Immunofluorescence images of COS1 cells transfected with N-terminally myc-tagged and C-terminally FLAG-tagged YBX3 constructs stained with FLAG-tag and myc-tag recognising antibodies and Hoechst.

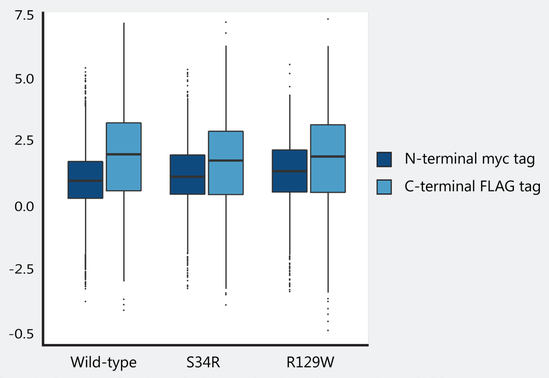

Figure 7. CellInsight analysis of the relative intensity difference between the nucleus and the cytoplasm in COS1 cells transfected with N-terminally myc-tagged and C-terminally FLAG-tagged YBX3 constructs. All values were normalised to the wild-type myc average value. A significant difference was found between the N- and C-terminal values within each group ($p<0.00001$).

DISCUSSION

The cleavage of YBX3 and independent localisation of its C-terminal peptide and the likely degradation of its N-terminal peptide have not been described previously. Our study suggest that the protein may have different roles depending on whether it is present in its complete or its cleaved form.

We have hereby demonstrated that both the S34R and the R129W variants in YBX3 have consequences on the proteins functional behaviour, but whether or not these changes are pathogenic or modifying is still to be determined.

FUTURE AIMS

To determine and unravel

- the cleavage- and nuclear localisation signals
- the function on differentiation in myoblasts
- the independent roles of the full length and cleaved protein in myoblasts
- the implications of the effects of the variants in myoblasts

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FUNDING

This work is funded by Muscular Dystrophy UK, the Sigrid Jusélius Foundation, the Finska Läkaresällskapet, the Medicinska understödsföreningen Liv och Hälsa, l'Association Française contre les Myopathies, and the Folkhälsan Research Center.

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