Localization of the adaptor protein Numb in close proximity to dihydropyridine receptors in skeletal muscle fibers

Rita De Gasperi[a,d,g], Zachary A. Graham[a,c], Lauren Harlow[a], William A. Bauman[a,b,c,e], Marco Brotto[g] and Christopher P. Cardozo[a,b,c,e,f].

[a]National Center for the Medical Consequences of Spinal Cord Injury and [b]Medical Service, James J. Peters VAMC, Bronx, NY; Departments of [c]Medicine, [d]Psychiatry, [e]Rehabilitation Medicine, [f]Pharmacologic Science and the [g]Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY. [g]Bone-Muscle Collaborative Sciences, College of Nursing & Health Innovation, The University of Texas at Arlington, Arlington, TX

1. ABSTRACT

Numb is a protein responsible for asymmetric division, cell fate commitment and vesicular trafficking. Numb has been shown to be vital to proliferation of Pax7+ satellite cells and for tissue repair after muscle injury. We have shown that Numb expression is increased in denervated skeletal muscle from animals treated with the synthetic androgen nandrolone and is associated with attenuation of Notch signaling present in denervated muscle. With these considerations in mind, the objective of the present study was to identify the cellular localization of Numb in skeletal muscle. Analysis of longitudinal sections of skeletal muscle by immunofluorescence identified a strong Numb immunoreactivity within muscle fibers that was organized in a pattern of linear staining traversing the fiber. A similar pattern of immunostaining was observed in individual, fixed muscle fibers from mouse hind limb muscles. To verify that this immunostaining represented intramyofibrillar Numb, tissues and dispersed muscle fibers were analyzed after genetic knockdown (KO) of Numb in transgenic mice developed by crossing HSA-MCM mice, which expressed ErT2-Cre under a human skeletal actin promoter, with mice containing floxed alleles for Numb and Numb-Like, a close relative of Numb with overlapping though distinct functions. Tamoxifen treatment reduced Numb expression by more than 50% in lysates of gastrocnemius, tibialis anterior, EDL and plantaris muscles, and by more than 90% in dispersed mouse hind limb muscle fibers. Numb immunoreactivity was also markedly reduced in fixed dispersed single muscle fibers from KO Numb mice, but still retained in the satellite cells population. Co-localization studies demonstrated that Numb does not co-localize with the Z-disk protein actinin but is close to, though not co-localized with, dihydropyridine receptors. These studies establish Numb as a bone fide skeletal muscle fiber protein, localized in the triad junction, which raise fascinating questions about its role(s) in skeletal muscle function and homeostasis.

2. PURPOSE

The purpose of this study was to determine the localization of Numb in adult skeletal muscle.

3. METHODS

- Immunofluorescence staining was performed using commercially available antibodies against the indicated proteins,. All experiments included sections stained with secondary antibody only.
- Unless otherwise indicated, imaging was performed using a Zeiss LSM 700 confocal microscope.
- Transgenic mice were bred and genotyped using standard procedures.
- Single muscle fibers were picked by hand after digestion of mouse calf muscle with collagenase. Fibers were washed to remove debris then fixed immediately or after culture for 48 hours, as indicated.

4. REFERENCES

1. Liu XH, Yao S, Qiao RF, Levine AC, Kirschenbaum A, Pan J, et al. Nandrolone reduces activation of Notch signaling in denervated muscle associated with increased Numb expression. Biochem Biophys Res Commun. 2011;414(1):165-9 . PMID: 22564549.

2. Liu XH, Yao S, Levine AC, Kirschenbaum A, Pan J, Wu Y, et al. Nandrolone, an anabolic steroid, stabilizes Numb protein through inhibition of mdm2 in C2C12 myoblasts. J Androl. 2012;33(6):1216-23. Epub 2012/06/16. doi: 10.2164/jandrol.112.016428. PubMed PMID: 22700758.

3. McCarthy JJ, Srikuea R, Kirby TJ, Peterson CA, Esser KA. Inducible Cre transgenic mouse strain for skeletal muscle-specific gene targeting. Skeletal muscle. 2012;2(1):8. Epub 2012/05/09. doi: 10.1186/2044-5040-2-8. PubMed PMID: 22564549

5. RESULTS

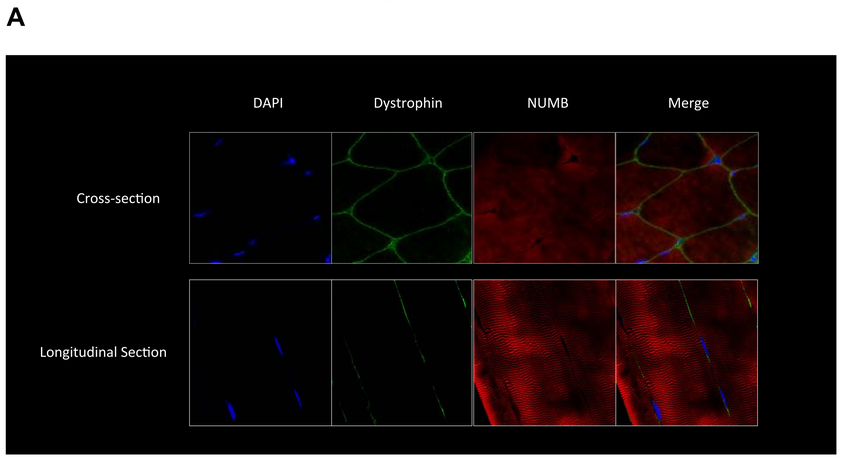

Fig. 1. Numb is present in mouse skeletal muscle fibers. Sections of mouse gastrocnemius muscle were immunostained with anti-Numb antibodies and anti-dystrophin antibodies then examined by confocal microscopy. Note the concentration of Numb within striations traversing the myofiber.

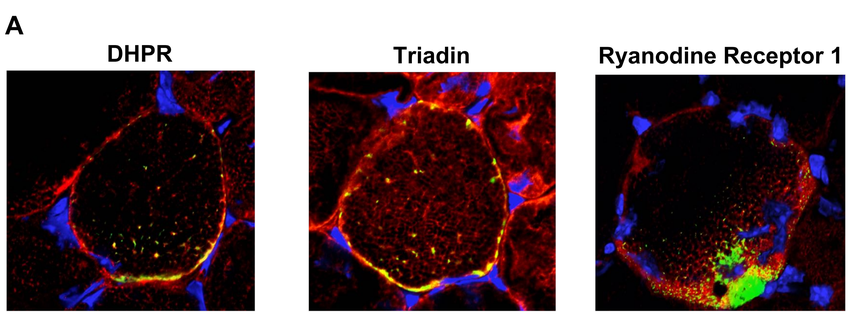

Fig. 2. Numb is found within muscle fbers near the triad. **A)** A vector expressing GFP-tagged Numb was introduced into mouse hindlimb muscle by electroporation. Fourteen days later, muscle was harvested and sections were stained with antibodies against triad proteins as indicated. In these images GFP-tagged Numb is green and the triad protein red. **B)** Single mouse hindlimb muscle fibers were immunostained with antibodies again Numb (green) and either dystrophin, actinin (a Z-disk protein) or dihydropyridine receptor (DHPR, a triad protein) (all of which are red) then imaged by confocal microscopy.

6. RESULTS CONT.

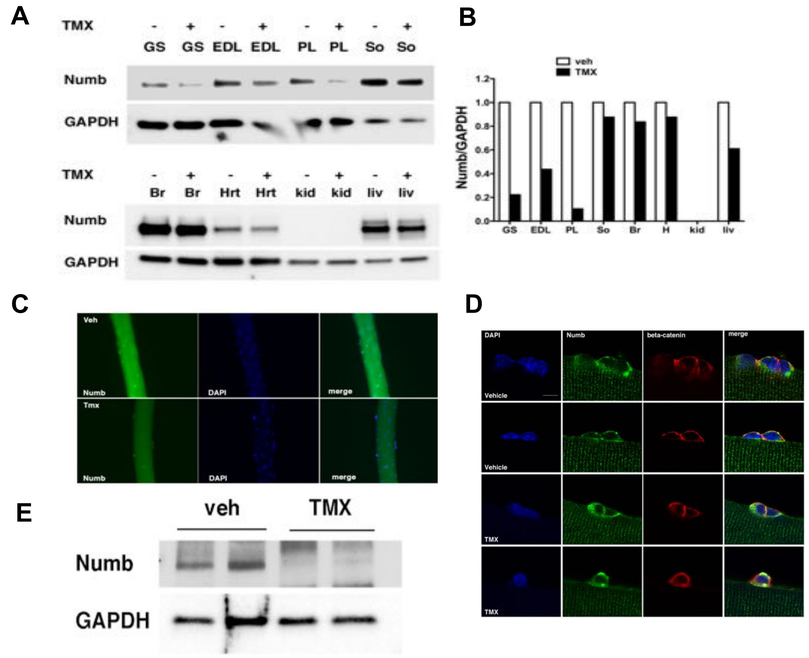

Fig. 3. Skeletal muscle-restricted Numb knockout reduces Numb immunostaining and protein levels in isolated myofibers. Mice expressing an ErT2 fusion protein under the human skeletal actin promoter (gift from Karyn Esser) homozygous for floxed Numb and NumbL alleles were treated with tamoxifen (TAM) or vehicle (VEH). **A)** Tissue Numb protein levels were assessed by Western blotting. **B)** Densitometry scanning was performed to quantify bands in A. **C)** Fluorescent microscopy was performed after immunostaining of single, fixed, isolated muscle fibers from mice treated with tamoxifen or vehicle. **D)** Muscle fibers were maintained in culture for 48 hours. Numb expression in activated satellite cells was assessed by immunostaining with antibodies against Numb and ß-catenin followed by confocal microscopy. **E)** Western blotting was performed to evaluate Numb protein levels in dispersed mouse hindlimb fibers.

7. CONCLUSIONS

- Adult skeletal muscle fibers contain Numb.
- Numb is concentrated within or very close to the triad.
- The findings suggest critical roles for Numb in excitation-contraction coupling through modulating calcium release or spatial relationship of the triad to sarcomeres.
- The data confirm the expected result that the HSA-MCM system does not result in recombination in resident satellite cells based on the robust Numb expression observed in satellite cells activated by culture of dispersed muscle fibers.

8. ACKNOWLEDGMENTS

Research support was provided by a VA RR&D Service Merit to CPC (B7756R) and a VA RR&D Service Center Award to WAB (B9212C).