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The hairpin region of WNT7A is sufficient for binding to the Frizzled7 receptor and to elicit signaling in myogenic cells

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ABSTRACT

Wnt signaling is essential for embryonic development and tissue homeostasis. So far, little is known about the importance and functional relevance of the different regions in WNT proteins including regions in their C-terminus identified as hairpin and linker. However, it was shown that the C-terminus of WNT7A comprising the linker and the hairpin region is sufficient to elicit signaling. Here, we demonstrate that actually the hairpin region of WNT7A in its C-terminus is fully sufficient to induce non-canonical signaling in myogenic cells while the linker region alone did not show biological activity. Of note, all known non-canonical signaling branches of WNT7A signaling in skeletal muscle were activated by the hairpin region of WNT7A thereby inducing hypertrophy in myotubes, symmetric expansion of satellite stem cells and migration of myoblasts. Furthermore, we demonstrate that the linker region in the C-terminus of WNT7A binds to the FZD7 receptor while it does not activate non-canonical Wnt signaling. However, the hairpin and the linker region of WNT7A can activate canonical Wnt signaling independent of each other suggesting that specificity of downstream signaling might be depending on those specific regions in the C-terminus.

1. Introduction

1.1. Molecular structure of WNT proteins

The family of WNT proteins contains 19 cysteine-rich, secreted ligands which bind to Frizzled (FZD) receptors located in the plasma membrane of target cells [25]. WNT proteins comprise between 350 and 400 amino acids and are subject to numerous post-translational modifications including disulfide-bonds, glycosylation and palmitoyleation [12,25,30]. The characteristic distribution of the 22 cysteines results in the formation of disulfide bonds required for proper folding of the protein [20,24,30]. Another common feature of all WNT proteins is their signal sequence, which was demonstrated to be essential for secretion [24]. Structurally, WNT proteins are composed of two domains, a N-terminal and a C-terminal domain [15]. While the N-terminal part contains 10 cysteine residues resulting in the formation of a cluster of alpha-helices, the C-terminus comprises twelve cysteine residues and a two-stranded beta-sheet [15]. For xenopus WNT8 (xWNT8) it was shown that the full length protein in a complex with the FZD receptor has the structure of a donut shape with two opposing sites containing regions termed as thumb and index fingers [15]. Of note, the main feature of the C-terminal region of xWNT8 which makes the index finger region is a 40 amino acid long beta-strand hairpin which is stabilized by a large amount of disulfide bonds. Interestingly, the C-terminal region of xWNT8 was shown to be sufficient for binding to FZD receptors. This structural information on xWNT8 is in line with a study demonstrating that the C-terminus of murine WNT7A containing the last 137 amino acids including the index finger region is sufficient to elicit Wnt signaling in a cell context dependent manner in mammalian cells [32]. This study also demonstrated that – at least for WNT7A – palmitoyleation is not required for binding to the FZD7 receptor and activation of the downstream signaling cascade. Subsequently, Agonstino et al. generated homology models of all murine and human WNT proteins showing that the vast majority of WNT proteins does not feature large insertions or deletions compared to xWNT8 [1]. Furthermore, the authors bioinformatically identified the thumb and index finger region in the WNT proteins to contribute significantly to the binding of the FZD receptors [1].

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1.2. Wnt signaling pathways

Wnt signaling can be divided into canonical and non-canonical signaling [25]. While canonical Wnt signaling requires the involvement of its critical component beta-catenin, non-canonical Wnt signaling signals independently of beta-catenin [24].

In the canonical signaling branch the WNT proteins bind to the respective member of the FZD receptor family and the low-density-lipoprotein-related proteins LRP5/6 (LDL receptor related protein) thereby activating Dishevelled, which leads to the inactivation of GSK3 (glycogen synthase kinase), a serine/threonine kinase. The inactivation of GSK3 then results in the blocking of phosphorylation of beta-catenin and thereby its stabilization [8]. Thereby beta-Catenin accumulates in the cytoplasm and translocates into the nucleus where it interacts with TCF/LEF (T cell factor/lymphoid enhancer factor family) to activate Wnt target genes [24,30].

While canonical Wnt signaling is depending on the transcriptional activity of beta-Catenin, non-canonical Wnt signaling does not require beta-Catenin activity. Non-canonical WNT proteins also signal through FZD receptors either in concert or independent of LRPs. Examples for non-canonical Wnt signaling pathways include the Wnt/Ca^{2+}, the PCP (planar cell polarity) signaling pathway and activation of the AKT/mTOR pathway [10,30].

1.3. Wnt signaling in skeletal muscle

Wnt signaling plays a crucial role during development and maintenance of skeletal muscle as well as for its regeneration [30]. WNT ligands control the expression of MRFs (myogenic regulatory factors) as well as the differentiation and self-renewal of satellite cells [13,19,30]. While the differentiation of satellite cells is mostly controlled by canonical Wnt signaling, self-renewal of satellite cells is regulated by non-canonical Wnt signaling, namely WNT7A [3,16,19]. Of note, WNT7A has a dual role in satellite cells. On the one hand, WNT7A increases the number of symmetric satellite stem cell divisions, on the other hand it enhances the directed migration of satellite cells [2,3,19]. Satellite stem cells are a subpopulation of satellite cells with a high engraftment potential which can divide either symmetrically or asymmetrically thereby giving rise to one satellite stem cell and one committed progenitor cell [18]. While WNT7A activates the planar cell polarity pathway (PCP) in satellite stem cells and thereby controls their division, the activation of Rho/Rac signaling in satellite cells enhances their migration. In myotubes and myofibers, however, WNT7A induces hypertrophy via activation of the AKT/mTOR pathway [29]. Of note, activation of the AKT/mTOR pathway is independent of the IGF-receptor thereby making WNT7A an interesting candidate for treatment of individuals suffering from cancer cachexia, the loss of skeletal muscle mass due to cancer. Indeed, recently it was shown that WNT7A counteracts cancer cachexia by activation of the AKT/mTOR pathway [26]. Remarkably, the receptor for WNT7A in myogenic cells is always FZD7 and signaling is strictly independent of beta-Catenin [2,3,19,29,32]. Although WNT7A always signals through FZD7 in skeletal muscle, the three downstream signaling pathways can be activated independently of each other; here the cell type determines which downstream signaling pathway is activated [2,19,29,30,32].

To investigate whether the whole C-terminus of WNT7A comprising the index finger (hairpin) region and the linker region is required to elicit signaling we generated different truncated versions of the C-terminus of WNT7A previously demonstrated to be fully biologically active. Here, we show that the hairpin of WNT7A is sufficient to activate all known downstream signaling pathways in myogenic cells including induction of hypertrophy, satellite stem cell divisions and migration. While the hairpin region is required for WNT7A mediated activation of non-canonical signaling, both the hairpin and the linker region of WNT7A can activate canonical Wnt signaling.

2. Material and methods

2.1. Cell Culture and transfection of cells

C2C12 myoblast were purchased from ATCC (CRL-1722) and maintained in DMEM (Sigma, 6046) supplemented with 10% FBS (fetal bovine serum, GibCo) and 2% Penicillin/Streptomycin (Thermo Fisher Scientific) in a tissue culture incubator at 37 °C, 5% CO_2 and 95% humidity. Differentiation into myotubes was induced by serum depletion, cells were cultured in DMEM (Sigma, 6046) containing 2% HS (horse serum, GibCo). LentiX cells were obtained from Björn von Eyss (Leibniz Institute on Aging) and maintained in DMEM (Sigma, 6046) supplemented with 10% FBS (GibCo) and 2% Penicillin/Streptomycin (Thermo Fisher Scientific). PC12 cells were cultured in DMEM supplemented with 10% FBS, while Ishikawa cells were grown in RPMI 1640 with glutamine (GibCo) and supplemented with 10% FBS (GibCo) [7,28]. Purified murine WNT7A-V2 (HQYARVWQCNCKFHWCCYVKCNTCS ERTEMYTCK) and WNT7A-V4 (TCWTTLPQFRELGYVLKDKY NEAVHVEPVRASRNKRPTFLKIKKPLSYRKPMDTD) peptides were obtained from genscript, the human full length WNT7A protein was obtained from R&D. Peptides and proteins were solubilized in 0.1% BSA in water and used in a final concentration of 100ng/ml if not indicated otherwise.

Transfection of cells was carried out using Lipofectamine 2000 (Thermo Fisher Scientific) in case of plasmid transfection and using Lipofectamine RNAiMax (Thermo Fisher Scientific) in case of transfection of siRNAs according to the manufacturer's protocol.

2.2. EDL myofiber culture

Preparation and cultivation of EDL (*extensor digitorum longus*) fibers was performed as described previously [26]. In brief, EDL muscles were isolated under sterile conditions and digested in 0.2% collagenase (from Clostridium histolyticum, Sigma) in DMEM (GibCo, 61965) at 37 °C in a water bath for 1.5 h. Dissociation of single myofibers was performed by trituration with sterile glass pipettes. Single isolated myofibers were cultured in myofiber culture medium (DMEM GibCo 61965, 20% FBS, 1% chicken embryo extract; United States Biological) at 37 °C, 5% CO_2 and 95% humidity for 42 h.

2.3. Generation of WNT7A conditioned medium

LentiX cells were cultured in either C2C12 growth media (DMEM, 10% FBS), C2C12 differentiation media (DMEM, 2% HS) or myofiber culture media (DMEM GibCo 61965, 20% FBS, 1% chicken embryo extract; United States Biological) for two days. The cell culture supernatant was removed and spun down at 870 g for 5 min to remove all living cells and cell debris. The supernatant was either directly used or frozen at −20 °C until further use.

For the experiments using the HA-blocking antibody the anti-HA antibody (Millipore, 05-904) was used in a final concentration 1 μg/ml, as a control a mouse IgG antibody (Millipore) was used also at a final concentration of 1 μg/ml.

2.4. Generation of cancer cachexia inducing conditioned medium

C26 cells were cultured in growth media (DMEM, 10% FBS) in a tissue culture incubator at 37 °C, 5% CO2 and 95% humidity. C26

cells were cultured in C2C12 cell differentiation media (DMEM, 2% HS) for two days. Media was filtered with a 0.45 μm syringe filter and ~50% of total volume of C2C12 differentiation media was replaced with C26 conditioned media at the respective day of the differentiation assay. Rapamycin (Cell Signaling, # 9904) was added to the cells at a final concentration of 20 μg/ml. The PI3Kinase inhibitor LY294002 (Cell Signaling, # 9901) was added to the cells at a final concentration of 50 μg/ml.

2.5. Scratch assay

Cells were plated and 24 h after plating, cells were treated with 50 μg/ml mitomycin-C (Millipore) for 2 h. Subsequently, the mono-layer of cells was scraped in a straight line. The plates were then extensively washed with culture medium and incubated for 24 h before analysis. Analysis was performed using DAPI staining after matching the reference points and enumeration of DAPI-stained nuclei in the scar was performed.

2.6. Immunostaining of cells

Cells were fixed with 2% PFA (paraformaldehyde) for 5 min at RT (room temperature), washed twice with PBS (pH 7.4), permeabilized with 0.1% Triton X-100 in PBS (pH 7.4) for 10 min at RT followed by incubation in blocking solution (5% HS in PBS (pH 7.4)) at RT for 1 h. Afterwards the respective antibodies were applied in blocking solution ON (over night) 4 °C. Then, cells were washed three times with PBS (pH 7.4) at RT and incubated with the respective secondary antibodies (Thermo Fisher Scientific) in blocking solution for 1 h at RT in the dark. After washing with PBS (pH 7.4) once, nuclei were counterstained with DAPI (50 μg/ml final concentration) and cells were washed three times with PBS (pH 7.4) for 5 min each at RT. Cells were then overlayed with PBS (pH 7.4) and directly used for microscopic image acquisition. The following antibodies were used: mouse anti-MYOSIN (undiluted, MF20; DSHB), mouse anti-MYOGENIN (undiluted, F5D, DSHB). Analyses were carried out using the Axio Observer.D1 (Carl Zeiss).

2.7. Immunostaining of single myofibers

Myofibers with their adjacent satellite cells were fixed with 2% PFA for 5 min at RT, washed three times with PBS (pH 7.4) and permeabilized with 0.1% Triton X-100 in PBS (pH 7.4) for 10 min at RT. Myofibers were then incubated with blocking solution (5% HS in PBS (pH 7.4)) for 1 h followed by incubation with the primary antibodies directed against PAX7 (undiluted, PAX7; DSHB) and GFP (1:1000, AbCam, #13970) at 4 °C ON. After three times washing with PBS (pH 7.4) for 5 min each, myofibers were incubated with the respective secondary antibodies (Thermo Fisher Scientific) for 1h at RT in the dark. Afterwards, myofibers were washed once with PBS (pH 7.4) for 5 min each followed by incubation with DAPI (50 μg/ml final concentration) for 5 min at RT, washed three times with PBS (pH 7.4) and then mounted in ProLong Gold anti-fade mounting medium (Thermo Fisher Scientific) on glass microscope slides. Analyses were carried out using the Axio Observer.D1 (Carl Zeiss). In brief, after 42h of culture satellite cell doublets (marked by the presence of PAX7) were counted as symmetric satellite stem cell divisions (both cells negative for YFP), symmetric committed progenitor cell divisions (both cells positive for YFP) or asymmetric satellite stem cell divisions (one cell positive for YFP, one cell negative for YFP). Then the percentage of symmetric satellite stem cell divisions was calculated and plotted.

2.8. Immunoblot analyses

Cells were lysed in RIPA buffer for 20 min on ice followed by sonication (10 cycles, 30 sec on and 5 sec off). Between 10 and 20 μg of protein were separated on a Bis-Tris protein gel. Then, a transfer to a nitrocellulose membrane (VWR) was carried out followed by incubation of the membrane in blocking solution (3% BSA in TBST) for 1 h at RT followed by the incubation with the respective primary antibodies in blocking solution at 4 °C ON. Afterwards, membranes were washed three times with TBST, incubated with the respective HRP-coupled secondary antibodies in blocking solution for 45 min at RT and washed 3 times with TBST. Membranes were incubated with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific).

The following primary antibodies were used:

HA (Millipore, 05-904 and Cell Signaling #3724), p-GSK3a/ b (Ser21/ 9) (NEB #9331), GSK3a/ b (NEB, 5676), active beta-CATENIN (NEB #8814), p-beta- CATENIN (Ser33/ 37/ Thr41) (NEB #9561), mouse anti-GAPDH (1:200, Santa Cruz, sc-365062).

The following secondary antibodies were used: goat anti-rabbit Immunoglobulins/HRP (1:1000, Dako, P0448), goat anti-mouse Immunoglobulins/HRP (1:1500, Dako, P0447).

For dot blot analysis 200 μl of cell culture supernatant was applied to a PVDF membrane followed by incubation with blocking solution (5% milk powder in TBS) for 1 h at RT and incubation with primary antibodies (AbCam) directed against the HA-tag ON at RT and the respective secondary antibodies for 1 h at RT.

2.9. Co-immunoprecipitation

Co-immunoprecipitation was carried out using beads coupled to GFP-trap agarose beads (Chromotek) according to the manufacturer's protocol.

2.10. TOP/FOP assay

The TOPFLASH assay was performed as previously described [23,32] using the SuperTop vector system and a luciferase kit (Promega). In brief, PC12 and Ishikawa cells were transfected with the respective expression plasmids and a TOPflash vector containing TCF/LEF-binding sites followed by the luciferase gene or a FOPflash vector containing mutated TCF/LEF-binding site as a negative control. Luciferase activity was assessed two days after transfection.

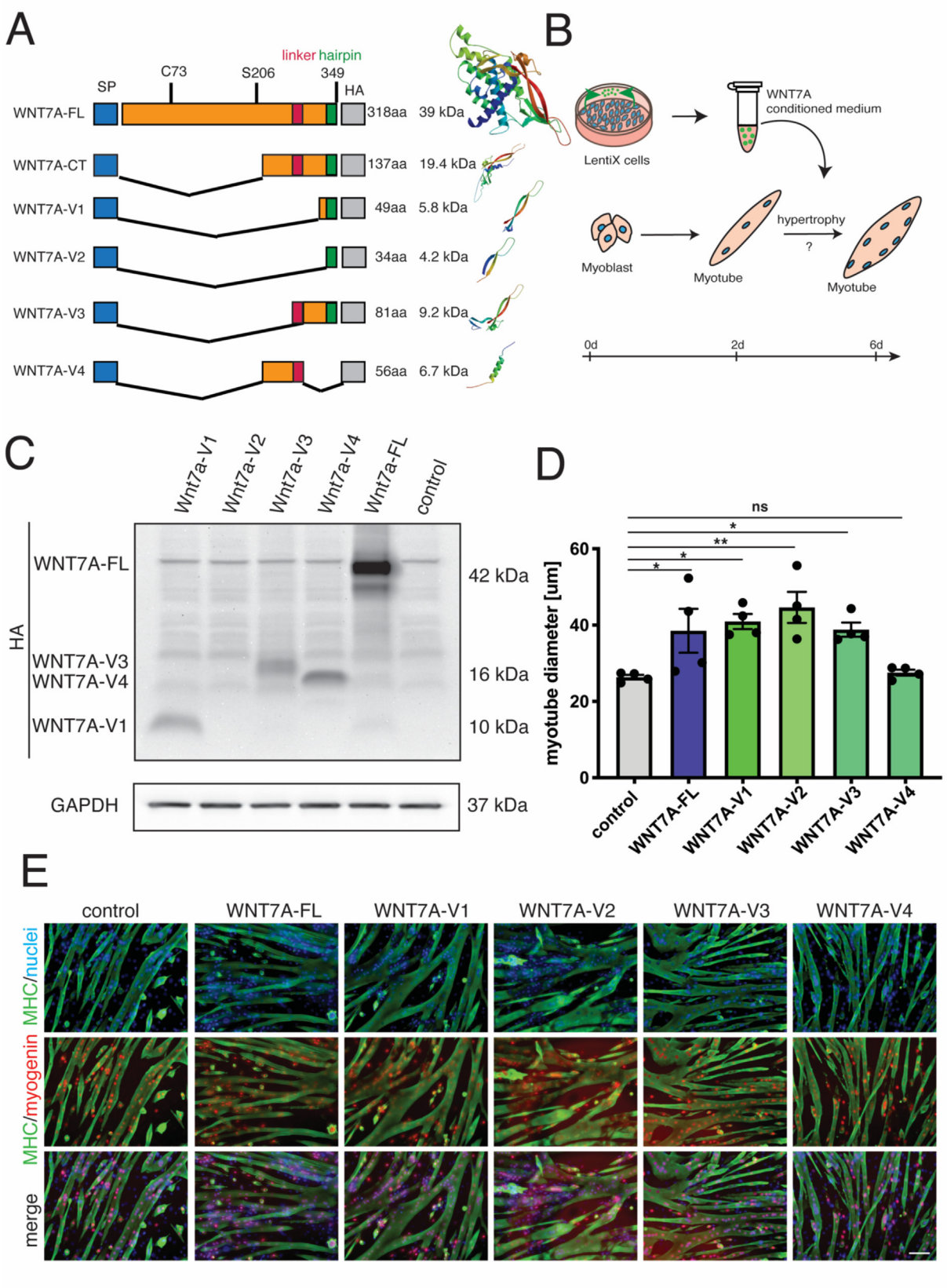
2.11. Statistical analysis

A minimum of 3 and a maximum of 4 replicates were analyzed for each experiment presented. Data are shown as SEM. Statistical significance was assessed by two-way ANOVA using Prism. A p value <0.05 were considered significant. A minimum of 5 fields of view were analyzed in cell culture experiments. For experiments involving EDL myofiber cultures at least 20 myofibers with their adjacent satellite cells were investigated per replicate per condition. The myotube diameter was measured as the average maximal myotube diameter of all myotubes marked by the expression of MYOSIN HEAVY CHAIN per condition. For each condition at least 3 replicates with 5 different fields of view were analyzed.

3. Results

3.1. The hairpin of WNT7A comprising the very last 34 aa is sufficient to induce hypertrophy in myotubes

First, we asked whether murine WNT7A variants comprising different parts of the WNT7A C-terminus were able to induce


A
linker hairpin
SP
C73
S206
349
HA
WNT7A-FL
318aa
39 kDa
WNT7A-CT
137aa
19.4 kDa
WNT7A-V1
49aa
5.8 kDa
WNT7A-V2
34aa
4.2 kDa
WNT7A-V3
81aa
9.2 kDa
WNT7A-V4
56aa
6.7 kDa
B
LentiX cells
WNT7A conditioned medium
Myoblast
Myotube
hypertrophy
?
Myotube
0d
2d
6d
C
Wnt7a-V1
Wnt7a-V2
Wnt7a-V3
Wnt7a-V4
Wnt7a-FL
control
HA
WNT7A-FL
WNT7A-V3
WNT7A-V4
WNT7A-V1
42 kDa
16 kDa
10 kDa
GAPDH
37 kDa
D
ns
*
**
*
*
myotube diameter [um]
60
40
20
0
control
WNT7A-FL
WNT7A-V1
WNT7A-V2
WNT7A-V3
WNT7A-V4
E
control
WNT7A-FL
WNT7A-V1
WNT7A-V2
WNT7A-V3
WNT7A-V4
MHC/nuclei
MHC/myogenin
merge

hypertrophy in C2C12 myotubes and which specific regions in the C-terminus are required. Beforehand it was shown that a truncated variant of WNT7A comprising the full C-terminus of WNT7A (WNT7A-CT) including the hairpin and the linker region was able to induce AKT/mTOR signaling and thereby hypertrophy in skeletal muscle [32]. To identify the region in the C-terminus of WNT7A essential to induce hypertrophy, we generated different C-terminal *Wnt7a* variants comprising either the hairpin (*Wnt7a-V1-3*) or the linker region (*Wnt7a-V4*). While *Wnt7a-V2* only comprised the codons encoding the hairpin, *Wnt7a-V1* and *Wnt7a-V3* contained the codons for the hairpin and additional N-terminally located amino acids of the C-terminus (Fig. 1A).

Since WNT proteins are secreted proteins which are integral for cell-to-cell communication [25], we expressed the different *Wnt7a* truncated versions in LentiX cells and harvested the conditioned medium containing the respective WNT7A variants (Fig. 1B). In brief, myoblasts were differentiated into myotubes for two days (Fig. 1B) and conditioned medium from LentiX cells containing the respective WNT7A variants – all containing a C-terminal HA tag – were added at day two of differentiation and cells were allowed to differentiate for additional four days (Fig. 1B). To demonstrate that the different WNT7A variants are indeed secreted by the LentiX cells and present in the conditioned medium, we first performed immunoblot analyses of conditioned media using antibodies directed against the HA-tag present in all constructs. Indeed, we detected WNT7A-V1, WNT7A-V3 and WNT7A-V4 in the conditioned media of LentiX cells while we were not able to detect the WNT7A-V2 variant in the conditioned medium by this method. We speculated that either the size of the WNT7A-V2 variant or the sample preparation for immunoblot analysis hindered its detection. Therefore, we performed a dot-blot of conditioned media from LentiX cells expressing the different *Wnt7a-HA* constructs without boiling the samples or adding denaturing reagents. Indeed, we detected all WNT7A-HA variants and the WNT7A-FL-HA protein in the respective conditioned media while no signals were obtained when conditioned medium from LentiX cells transfected with a *YFP* control plasmid were used (Fig. S1A). Interestingly, we found that addition of conditioned media containing the WNT7A variants WNT7A-V1-3 but not WNT7A-V4 induced hypertrophy of already formed myotubes as evidenced by an increased myotube diameter (Fig. 1D-E) further suggesting that also WNT7A-V2 is secreted by LentiX cells into the culture medium. Furthermore, we demonstrate that the hairpin present in the variants WNT7A-V1-V3 but not the linker region only present in WNT7A-V4 is required for full biological activity of WNT7A in myotubes. To unequivocally show that the WNT7A-V2 peptide induces hypertrophy, we added purified WNT7A-V2 and WNT7A-V4 peptides as well as WNT7A full length protein (100ng/ml final concentration) to myotubes at day 2 of differentiation and assessed the myotube diameter at day 6 of differentiation. Indeed, we could demonstrate that the WNT7A-V2 peptide induces hypertrophy to a similar extent as the WNT7A full length protein while the WNT7A-V4 peptide did not show activity (Fig. S1B). Next, we tested whether the WNT7A-V2 peptide induces hypertrophy in C2C12 cells in a dose-dependent manner. We show that the WNT7A-V2 induces hypertrophy in a dose-dependent manner and that even 10ng/ml resulted in a significant increase in myotube diameter while lower amounts only induced a slight increase in myotube diameter and higher amounts did not result in even more increased myotube diameters (Fig.S1C). To investigate whether the whole hairpin is required for biological activity, we generated truncated versions of the hairpin and tested whether those ones are sufficient to induce hypertrophy in myotubes. However, none of the truncated hairpin variants (WNT7A-NANO1-3) induced hypertrophy suggesting that the full hairpin is required for biological activity (Fig. S1D).

To unequivocally demonstrate that the hairpin region of WNT7A is sufficient to induce hypertrophy, we used blocking antibodies directed against the HA-tag present in all WNT7A variants in combination with conditioned media containing the respective WNT7A-HA variants (Fig. S2A). In brief, C2C12 myotubes were differentiated for three days followed by addition of conditioned media containing the different WNT7A-HA variants together with the HA blocking antibody or a control antibody. At day 5 of differentiation the myotube diameter as a measure of hypertrophy was assessed. Indeed, we found that the HA blocking antibody completely augmented hypertrophy induced by the WNT7A-HA variants V1-V3 suggesting that all variants including WNT7A-V2-HA are secreted by LentiX cells and present in the conditioned medium (Fig. S2A). Of note, when we used purified WNT7A-V2 peptides or WNT7A-FL proteins without an HA-tag, the HA blocking antibody did not augment hypertrophy (Fig. S2B).

Previously, we demonstrated that full length WNT7A protein counteracts myotube atrophy caused by cancer cachexia [26]. Here, we tested whether the hairpin of WNT7A (Wnt7a-V2) was also able to counteract myotube atrophy in cancer cachexia conditions. Therefore, we generated conditioned medium from C26 colon carcinoma cells, known to induce cancer cachexia (Fig. S1E) [26]. C2C12 myoblasts were differentiated into myotubes followed by incubation with cancer cachexia inducing C26 conditioned medium and conditioned medium containing the respective WNT7A variants (Fig. S1E). Of note, we observed that all WNT7A variants containing the hairpin (WNT7A-V1-V3) counteracted myotube atrophy while the WNT7A variant only containing the linker region (WNT7A-V4) did not display any effect on myotube size (Fig. S1F).

3.2. The hairpin of WNT7A signals through the AKT/mTOR pathway thereby inducing hypertrophy

Next, we asked whether the hairpin of WNT7A induces hypertrophy of myotubes through activation of the AKT/mTOR pathway as previously shown for the WNT7A full length protein and the whole C-terminus of WNT7A [29,32]. In brief, we used conditioned media containing the different WNT7A variants comprising the hairpin regions of WNT7A in combination with rapamycin, a known inhibitor of mTOR and added rapamycin and the WNT7A variants simultaneously to differentiated myotubes and assessed the myotube diameters at day 5 of differentiation (Fig. 2A). While all WNT7A variants containing the hairpin region (WNT7A-V1-V3) induced hypertrophy in the absence of rapamycin, rapamycin fully inhibited myotube hypertrophy in those conditions (Fig. 2B-C) sug-

Fig. 1. The hairpin of WNT7A is sufficient to induce hypertrophy in myotubes. A) Schematic showing the different WNT7A variants used and their respective theoretical molecular weight and molecular structure. SP: signal peptide, HA: hemagglutinin. FL: full length, CT: C-terminus. The models of the different WNT7A variants were generated using the SWISS-MODEL report system from the Biozentrum at the University of Basel [4,5,6,11]. B) Experimental scheme depicting the hypertrophy assay with conditioned medium containing the different WNT7A variants. d: days. C) Immunoblot analysis of conditioned medium from transiently transfected LentiX cells using antibodies directed against the HA-tag demonstrating that the different WNT7A variants are secreted to the culture medium. D) C2C12 cells were incubated with conditioned medium containing the different WNT7A variants at day 4 of differentiation and differentiated for 2 additional days. The myotube diameter was assessed at day 6 of differentiation. n = 4. Two-way ANOVA, *=p < 0.05, **=p < 0.01, ns: not significant. E) Representative images of the quantification shown in D). MYOSIN HEAVY CHAIN (MHC) in green, MYOGENIN in red, nuclei in blue. Scale bar: 100 μm.

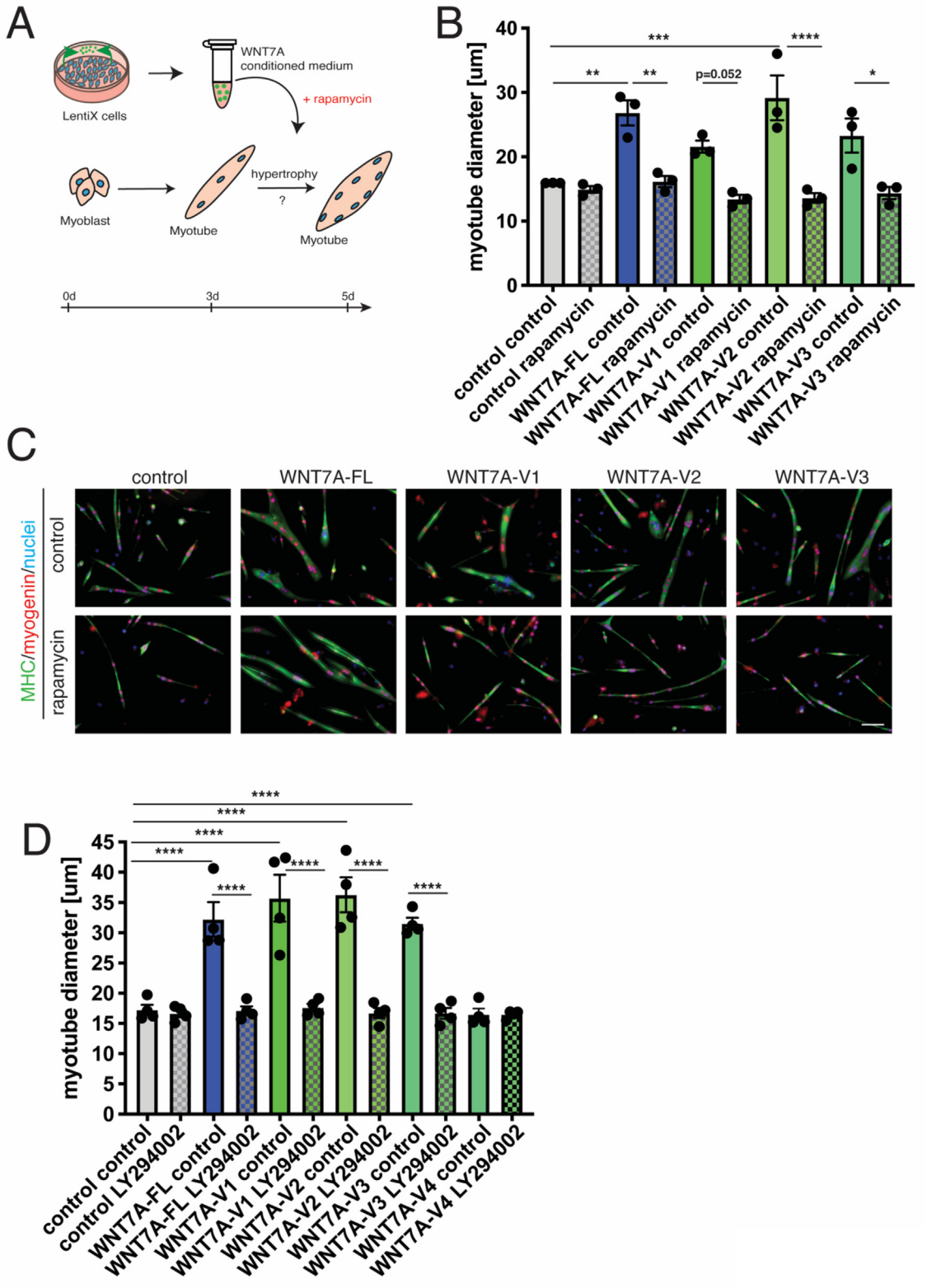


Fig. 2. Induction of hypertrophy by WNT7A variants containing the hairpin region is depending on the AKT/mTOR pathway. A) Experimental scheme depicting the hypertrophy assay and inhibition of mTOR by rapamycin with conditioned media containing the different WNT7A variants. d: days. B) C2C12 cells were incubated with conditioned media containing the different WNT7A variants and rapamycin or a control at day 3 of differentiation and differentiated for 2 additional days. The myotube diameter was assessed at day 5 of differentiation. n = 3. Two-way ANOVA, *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001, ns: not significant. C) Representative images of the quantification shown in D). MYOSIN HEAVY CHAIN (MHC) in green, MYOGENIN in red, nuclei in blue. Scale bar: 100 μm. D) C2C12 cells were incubated with conditioned media containing the different WNT7A variants and LY294002 or a control at day 2 of differentiation and differentiated for 4 additional days. The myotube diameter was assessed at day 6 of differentiation. n = 4. Two-way ANOVA, *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001, ns: not significant.

gesting that the hairpin of WNT7A induces hypertrophy via activation of the AKT/mTOR pathway. Of note, hypertrophy induced by the WNT7A variants V1-3 was also inhibited when we blocked PI3Kinase activity using LY294402 (Fig. 2D) further supporting our notion that the WNT7A variants V1-V3 signal through the AKT/mTOR pathway.

3.3. The hairpin of WNT7A binds to the FZD7 receptor in myotubes

Since the full length WNT7A protein and the C-terminus of WNT7A were shown to bind to the FZD7 receptor in myotubes thereby inducing hypertrophy via the AKT/mTOR pathway [29,32], we investigated if the different WNT7A variants comprising the hairpin or the linker region bind to the FZD7 receptor. Briefly, we either expressed *Fzd7*-YFP or *YFP* (yellow fluorescent protein) with the different *Wnt7a* variants (all comprising a C-terminally located HA-tag) in C2C12 myoblasts and performed co-immunoprecipitation experiments using antibodies directed against GFP (green fluorescent protein) which also react with the YFP-tag followed by immunoblot analysis for the HA-tag. Indeed, the hairpin containing variants WNT7A-V1 and V3 co-immunoprecipitated with FZD7 in myogenic cells (Fig. 3A and Fig. S3). However, also here we were not able to detect WNT7A-V2 using immunoblot analyses, neither in the input control nor in the immunoprecipitated samples. Interestingly, we found that WNT7A-V4 only containing the linker region of WNT7A also co-immunoprecipitated with FZD7 suggesting that the linker region and the hairpin region both have the ability to bind to FZD7. Next, we asked whether FZD7 is required for induction of hypertrophy mediated by the WNT7A hairpin. In brief, we differentiated myoblasts into myotubes for 2 days followed by a transfection with small interfering RNAs (siRNAs) targeting *Fzd7*. Simultaneously with siRNA transfection we added conditioned media containing WNT7A-V2 comprising only the hairpin region or full length WNT7A protein including the linker region (Fig. 3B). Transfection of *Fzd7* siRNA fully abrogated the ability of the hairpin containing WNT7A-V2 fragment to induce hypertrophy, when added as part of the conditioned medium from LentiX cells or as a purified peptide (Fig. 3C-E). Thus, we conclude that the hairpin of WNT7A is sufficient to induce hypertrophy via the FZD7 receptor.

3.4. The hairpin of WNT7A drives symmetric division of satellite stem cells

WNT7A-FL and WNT7A-CT drive the symmetric expansion of satellite stem cells in concert with their receptor FZD7 through activation of the PCP signaling pathway [19,32]. Consequently, we asked whether the hairpin or the linker region of WNT7A is required to induce the symmetric division of satellite stem cells. Therefore we isolated single EDL myofibers with their adjacent satellite cells from ROSA-YFP;myf5-cre mice, a reporter mouse model allowing the distinction of satellite stem cells and committed progenitor cells due to lineage expression of YFP [18]. While satellite stem cells are YFP-lineage negative (PAX7+/YFP-) and are required for maintaining the stem cell pool, committed progenitor cells expressing YFP (PAX7+/YFP+) undergo terminal myogenic differentiation [18,27]. Satellite stem cells can divide either symmetrically giving rise to two satellite stem cells (YFP negative) or asymmetrically giving rise to one satellite stem cell (YFP negative) and one committed progenitor cell (YFP positive). While the symmetric divisions are typically planar divisions, asymmetric divisions are apical-basal in respect to the myofiber (Fig. 4A).

Interestingly, when we isolated single myofibers with their adjacent satellite cells from ROSA-YFP;myf5-cre reporter mice and cultured them for 42 h in the presence of cell culture supernatants containing the different WNT7A variants (Fig. 4A), we found that the variants WNT7A-V1-V3 comprising the hairpin of WNT7A drove symmetric expansion of satellite stem cells (PAX7+/YFP-) while WNT7A-V4 did not affect satellite stem cell divisions (Fig. 4B). Of note, the increase in planar satellite stem cell divisions for the variant WNT7A-V2 comprising only the hairpin region of WNT7A was confirmed in an independent experiment (Fig. S4A) and when the purified WNT7A-V2 peptide was used (Fig. S4B). These data suggest that the hairpin region of WNT7A is sufficient to induce satellite stem cell divisions, most likely through activation of the PCP signaling pathway in satellite stem cells. To test whether induction of symmetric satellite stem cell divisions is depending on FZD7, we used a siRNA based approach targeting *Fzd7*. In brief, we isolated single myofibers with their adjacent satellite cells from ROSA-YFP;myf5-cre mice and transfected them with a siRNA targeting *Fzd7* after 4 h of culture, a time point when the basal lamina surrounding the myofiber and the satellite cells is permeable for siRNAs [14]. At the time of transfection we also added the respective conditioned media containing the different WNT7A variants. Transfection of *si-Fzd7* fully abrogated the increase in symmetric satellite stem cell divisions by WNT7A-FL and WNT7A-V2 (Fig. 4C) suggesting that the hairpin of WNT7A is signaling through the FZD7 receptor to induce symmetric expansion of satellite stem cells.

3.5. The hairpin of WNT7A induces migration in myoblasts

WNT7A in concert with FZD7 polarizes myogenic cells and stimulates their cell migration through activation of RHO and RAC [2]. Hence, we wondered whether the hairpin of WNT7A is also sufficient to induce migration in this cellular context or if signaling occurs via the linker region of WNT7A. Therefore, we performed scratch assays of C2C12 myoblasts in the presence of the different WNT7A variants (Fig. 4D). To exclude any potential effects of altered rates of cell proliferation, we treated the cells with mitomycin-C at the time of scratching. Addition of conditioned media with WNT7A variants containing the hairpin of WNT7A increased the migration of myoblasts while conditioned media containing only the linker region of WNT7A (WNT7A-V4) did not enhance migration (Fig. 4E). Of note, also the purified WNT7A-V2 peptide induced migration of C2C12 cells while the WNT7a-V4 peptide did not affect migration of C2C12 cells (Fig. S4C) supporting our notion that the hairpin of WNT7A is required to induce migration of myoblasts.

3.6. The hairpin and the linker region of WNT7A activate canonical Wnt signaling

We demonstrated that the hairpin of WNT7A is required for the induction of non-canonical Wnt signaling. However, we observed binding of the hairpin as well as the linker region to FZD7 in myogenic cells. This made us speculate if activation of canonical Wnt signaling is depending on the hairpin or the linker region suggesting that the binding of the respective region in WNT7A decides on the signaling pathway to be activated. WNT7A is a good candidate to test for this since it is able to induce non-canonical and canonical Wnt signaling in a cell context dependent manner [32]. Therefore, we assessed the activation of the beta-catenin gene reporter TopFlash in PC12 and Ishikawa cells, two cell lines known for activation of canonical Wnt signaling by WNT7A. Briefly, we transfected PC12 cells with the respective *Wnt7a* constructs or added the respective WNT7A peptides to Ishikawa cells and assessed activation of canonical Wnt signaling. Notably, the hairpin as well as the linker region of WNT7A induced the beta-catenin gene reporter TopFlash in PC12 and Ishikawa cells (Fig. 5A-B). We then tested if phosphorylation of GSK3 in Ishikawa cells could be induced by the hairpin region of WNT7A as previously shown for the WNT7A-CT

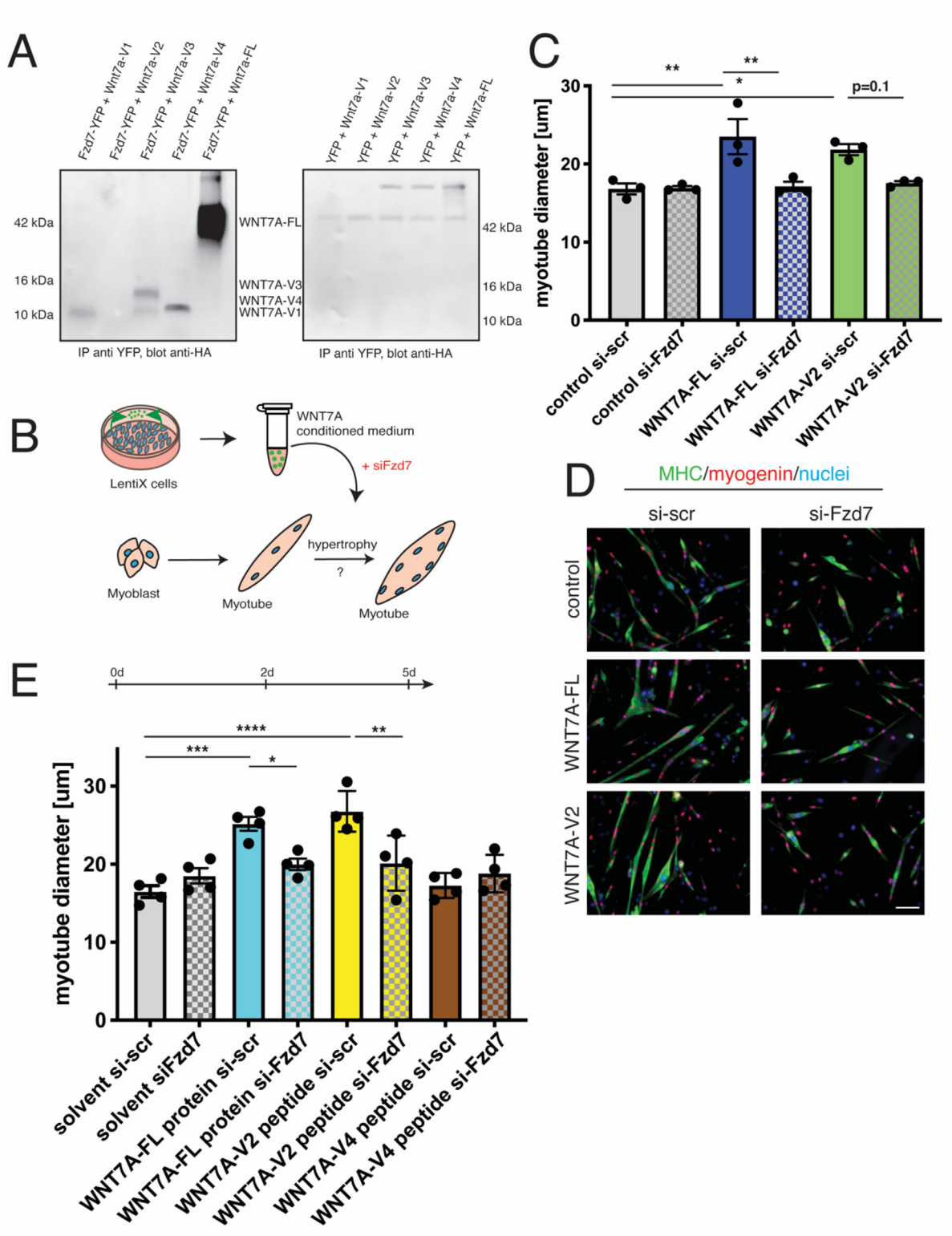


Fig. 3. FZD7 is the receptor for the hairpin region of WNT7A and FZD7 is required to induce hypertrophy by the hairpin region. A) Co-immunoprecipitation analysis demonstrates that the different WNT7A variants bind FZD7-YFP. In brief, C2C12 cells were transfected with the respective constructs harvested two days after transfection. A construct only containing the YFP-tag was used as a negative control. B) Experimental scheme depicting the hypertrophy assay and knockdown of *Fzd7* using *siFzd7* or *siscr* as control with conditioned media containing the different WNT7A variants. d: days. C) C2C12 cells were incubated with conditioned media containing the different WNT7A variants and transfected with *siFzd7* or *siscr* as a control at day 2 of differentiation and differentiated for 3 additional days. The myotube diameter was assessed at day 5 of differentiation. n = 3. Two-way ANOVA, *=p < 0.05, **=p < 0.01. D) Representative images of the quantification shown in C). MYOSIN HEAVY CHAIN (MHC) in green, MYOGENIN in red, nuclei in blue. Scale bar: 100 μm. E) C2C12 cells were incubated with different WNT7A peptides or the WNT7A full length protein and transfected with *siFzd7* or *siscr* as a control at day 2 of differentiation and differentiated for 4 additional days. The myotube diameter was assessed at day 6 of differentiation. n = 4.Two-way ANOVA, *=p < 0.05, **=p < 0.01, ***p = 0.001, ****=p < 0.0001.

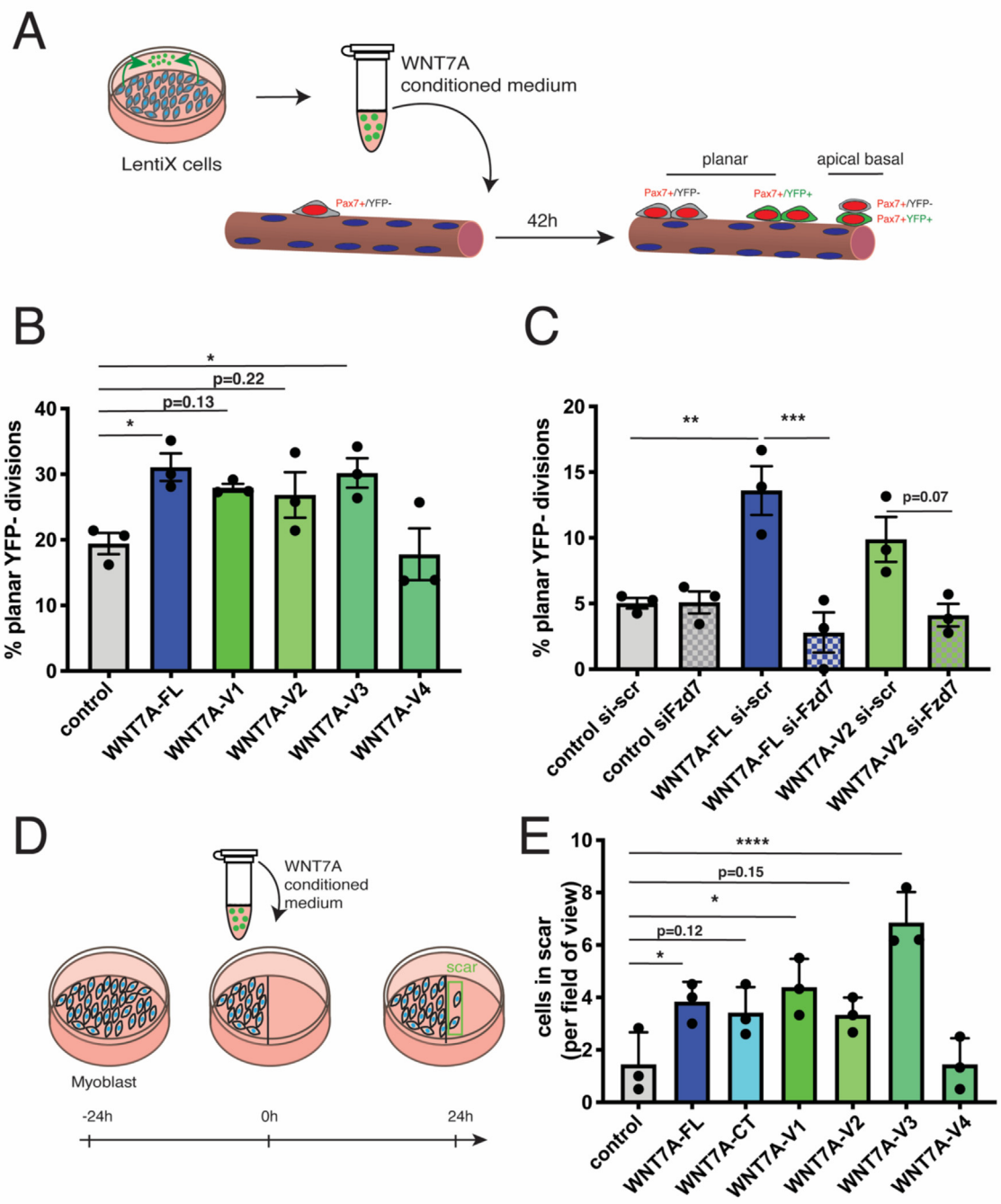


Fig. 4. A WNT7A variant only containing the hairpin region drives symmetric expansion of satellite stem cells and induces migration. A) Experimental scheme depicting the expansion of satellite stem cells with conditioned media containing the different WNT7A variants. h: hours. B) WNT7A variants V1-V3 stimulate the symmetric expansion of satellite stem cells (marked by being YFP lineage negative using ROSA-YFP;myf5-cre reporter mice) after 42 h of culture. n = 3. Two-way ANOVA, *=p < 0.05. C) Stimulation of symmetric expansion of satellite stem cells by WNT7A-FL and WNT7A-V2 is depending on *Fzd7* expression. Transfection with *siFzd7* or *siscr* as control was carried out 4 h after isolation of single myofibers with their respective satellite cells followed by an additional 38 h of culture (42 h culture time in total). n = 3. Two-way ANOVA, **=p < 0.01, ***=p < 0.001. D) Experimental scheme depicting the analysis of migration of C2C12 cells after incubation with the different WNT7A variants. C2C12 cells were seeded and after 24 h of culture the cells were incubated with mitomycin to inhibit cell proliferation. Then one half of the culture plate was scraped and the different WNT7A conditioned media were added. The number of cells in the scar was assessed 24 h after scraping. E) WNT7A-V1-V3 induce migration of C2C12 cells in a scratch assay. n = 3. Two-way ANOVA, *=p < 0.05, ***=p < 0.001.

variant [32]. Indeed, all WNT7A variants comprising the hairpin region as well as WNT7A-V4 comprising the linker region were able to slightly induce phosphorylation of GSK3 in Ishikawa cells (Fig. 5C-F, Fig. S5). Therefore, we suggest that induction of non-canonical Wnt signaling is only depending on binding of the hairpin region of WNT7A while induction of canonical Wnt signaling can be induced by binding of the hairpin or the linker region of WNT7A.

4. Discussion

In summary, this work demonstrates that the hairpin of WNT7A located at the very last amino acids in the C-terminus is required and sufficient for the activation of non-canonical Wnt signaling, while the hairpin as well as the linker region can induce canonical Wnt signaling.

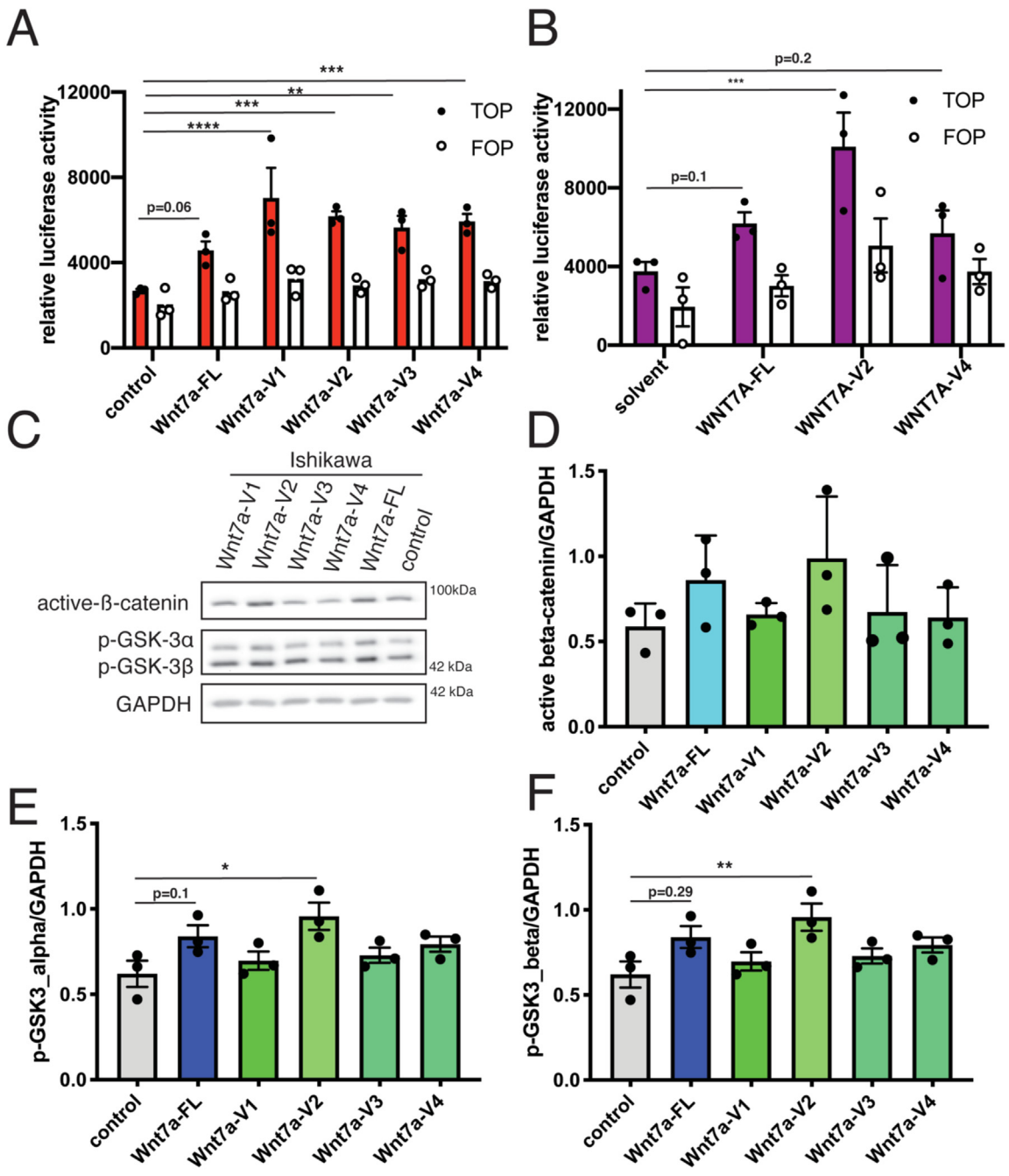


Fig. 5. WNT7A variants either containing the hairpin or the linker region activate canonical Wnt signaling in a cell context dependent manner and lead to phosphorylation of GSK3. A) Transfection of the different *Wnt7a* constructs into PC12 cells promotes activation of the beta-catenin TopFlash gene reporter. Luciferase activity was measured 2 days after transfection and normalized to protein amounts. n = 3. Two-way ANOVA, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001. B) Addition of different WNT7A peptides to Ishikawa cells promotes activation of the beta-catenin TopFlash gene reporter. Luciferase activity was measured 2 days after addition of the respective peptides. n = 3. Two-way ANOVA, ***=p < 0.001. C) Ishikawa cells were transfected with the different *Wnt7a* constructs followed by immunoblot analysis for active beta-catenin, phosphorylated GSK-3alpha and beta. GAPDH was used as a loading control. D) Quantification of C for beta-catenin normalized to GAPDH levels, n = 3. Two-way ANOVA. E) Quantification of C) for phosphorylated GSK-3alpha normalized to GAPDH, n = 3. Two-way ANOVA, *=p < 0.05. F) Quantification of C) for phosphorylated GSK-3beta normalized to GAPDH, n = 3. Two-way ANOVA, **=p < 0.01.

Most studies investigating the different regions of the WNT proteins analyzed the binding or potential binding of WNT fragments to the FZD receptors [9,15]. Here, we demonstrate that the hairpin region of WNT7A is fully sufficient to activate non-canonical and canonical Wnt signaling. Interestingly, we found that non-canonical Wnt signaling can only be elicited by the hairpin region suggesting that this region is a defining factor between canonical and non-canonical Wnt signaling. This also let us speculate that the full length WNT7A protein has multiple binding regions to its receptor FZD7 in myogenic cells consistent with structural analysis of xWNT8 binding to the FZD8 receptor [15]. The authors show that xWNT8 comprises an index finger and a thumb region in the C-terminus of xWNT8 and that both regions bind to the receptor FZD8. The decision which signaling pathway is activated after

binding of WNT7A to the FZD7 receptor in myogenic cells depends on the cellular context, here the differentiation status of the cells [2,19,29]. Wnt signaling is known to be regulated by gradients of *Wnt* expression, e.g. during embryonic development [21] as well as through WNT binding proteins such as SRFs (serum response factor) thereby forming a non-functional complex [22]. A recent study on WNT7A and WNT7B by Eubelen et al. [9] suggests that the intrinsically disordered region of WNT7 binds Reck and that the availability of Reck-bound WNT7 to induce FZD7 receptor mediated signaling depends on the interaction of GPR124 and DSHV. This work further suggests that the selective WNT7 recognition by different FZD receptors is controlled by Reck and the availability of "free" WNT7 to bind to FZD7. If those binding proteins selectively bind the hairpin or the linker region of WNT proteins needs to be investigated in the future as well as the question if binding of those factors decides on the signaling pathway to be activated.

WNT proteins are promising candidates for therapeutic interventions, e.g. WNT7A was shown to ameliorate muscular dystrophy and prevent cancer cachexia induced atrophy of skeletal muscle [26,31]. However, the dispersion of full length WNT proteins in the tissue is limited due to the modification with monounsaturated palmitoleate [17]. For *Wnt7a* it was shown that the C-terminus without palmitoyleation sites is sufficient for full functionality [32]. Nevertheless, tissue dispersion is still limited and the size of the respective peptide comprising ~20kDA (Fig. 1A) is still considerable high hampering the use of WNT7A as a therapeutic agent. The demonstration that a WNT7A peptide comprising only ~4-5kDA in size as the WNT7A-V2 variant makes the use of a WNT7A based therapy for different muscle wasting conditions such as cancer cachexia more likely. If small fragments of other WNT proteins comprising the hairpin region in the C-terminus are also fully functional, needs to be demonstrated. However, different studies demonstrate that WNT proteins are structurally highly conserved in this region [1] and that a C-terminal fragment of xWNT8 was able to bind to FZD8 [15] suggesting that the hairpin region in all WNT proteins is of critical importance.

Our work demonstrates for the first time that the hairpin region of WNT proteins, here WNT7A, is fully sufficient and required to elicit signaling. This opens important new avenues for the development of WNT7A-based peptide mimetics for the treatment of muscle wasting diseases, e.g. cancer cachexia or Duchenne muscular dystrophy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

MS, CP, CJ and JvM designed and performed most experiments, analyzed data and interpreted results. MS and JvM analyzed data and interpreted results. JvM wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.10.047.

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