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#### **BRIEF REPORT**



# Positive cooperativity in synergistic activation of Wnt proteins

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#### Abstract

**Background** Wnt proteins are crucial for embryonic development, stem cell growth, and tissue regeneration. Wnt signaling pathway is activated when Wnt proteins bind to cell membrane receptors.

**Methods and results** We employed a luciferase reporter assay in HEK293STF cells to measure Wnt protein-induced signaling. We observed that Wnt3a uniquely promotes the Wnt/β-catenin pathway through positive cooperativity. Additionally, MFH-ND, a molecular mimic of Wnt ligands, markedly increased Wnt3a-induced signaling in a dose-responsive manner. This suggests that various Wnt ligands can synergistically enhance Wnt pathway activation.

**Conclusions** The study suggests the likelihood of various Wnt ligands coexisting in a single signalosome on the cell membrane, providing new insights into the complexities of Wnt signaling mechanisms.

Keywords Cooperative binding · Signalosome · Wnt3a · Wnt mimic · Wnt/b-catenin signaling

#### Introduction

Wnt/ $\beta$ -catenin signaling pathways play a crucial role in embryonic development, as well as in stem cell proliferation, differentiation, self-renewal, tissue regeneration, and remodeling in adults [1, 2]. In the absence of Wnt signaling, phosphorylated  $\beta$ -catenin undergoes continuous proteasomal degradation. This degradation is facilitated by a destruction complex consisting of adenomatous polyposis coli, Axin, casein kinase 1 $\alpha$ , and glycogen synthase kinase 3 $\beta$  [3]. Activation of Wnt/ $\beta$ -catenin signaling occurs when Wnt proteins bind to both Frizzled (FZD) and low-density lipoprotein receptor-related proteins 5 or 6 (LRP5/6) transmembrane receptors simultaneously [4]. This activation leads to an increase in intracellular  $\beta$ -catenin levels, resulting in alterations in gene expression and cellular behavior [5].

Wnt proteins, being lipid-modified, possess hydrophobic properties and are relatively insoluble [6]. Consequently, their range of action is limited, but they exhibit potent morphogenic effects. Notably, the concentration gradient of Wnt proteins within the stem cell niche plays a crucial role in embryonic development and stem cell renewal in vivo [7]. However, the detailed correlation between extracellular Wnt protein concentration and intracellular Wnt signaling activity remains unexplored. Furthermore, similar to other cellular signaling pathways, binding of Wnt proteins to their receptors, FZDs and LRP5/6 can induce the formation of signalosomes-large transmembrane oligomer complexes [8]. Yet, the impact of Wnt signalosome formation on the strength of Wnt signaling activity within cells remains unclear. Additionally, there are nineteen Wnt proteins in humans and other mammals [5, 6], and they are often expressed simultaneously [9, 10]. The synergistic effects of different Wnt proteins in organ development [10] and intracellular signaling [11] have been reported [12–14]. Nevertheless, the interplay between different Wnt proteins and its influence on Wnt signalosome formation and intracellular Wnt activity remain unclear.

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#### Results

In the current study, we quantified the Wnt signaling activity induced by Wnt proteins using a luciferase reporterbased cellular assay in HEK293STF cells. These cells are a stably transfected HEK293 cell line expressing a luciferase reporter controlled by seven LEF/TCF binding sites (Super TOP-Flash) [15].

To induce Wnt activity, we treated the HEK293STF cells with recombinant Wnt3a. Upon analyzing the Wnt activity within the cells by measuring luciferase activity, we observed that the Wnt activation induced by Wnt3a was not a single-phase response (Figs. 1A, B). Curve fitting analysis revealed a calculated Hill factor of  $3.5 \pm 0.5$ 

(Fig. 2). A Hill factor greater than 1.0 signifies positive cooperation [16], our data indicates strong positive cooperation in Wnt activation by Wnt3a within the cells.

To investigate if the synergistic activity of different Wnt proteins exhibits positive cooperation, we employed a Wnt mimic small-molecule called MFH-ND, as there was a lack of reliable recombinant canonical Wnt proteins apart from Wnt3a. MFH-ND was designed by combining a small molecule, MFH, which binds to the cysteine-rich domain (CRD) of FZD, with a peptide derived from the N-terminal of Dickkopf-1 (DKK1) protein, named ND, which binds to the first  $\beta$ -propeller domain of LRP5/6. This construct enables MFH-ND [17] to mimic Wnt proteins in a broad spectrum manner.

Although MFH-ND exhibits broad binding capabilities to different FZD-CRDs and the first  $\beta$ -propeller domains



Fig. 1 Wnt activation in HEK293STF cells measured by TOP-Flash assay. A Luciferase-based TOP-Flash assay demonstrating the binding capacity of Wnt3a, MFH-ND, and the combination of Wnt3a+MFH-ND at increasing concentrations. Results show the mean  $\pm$  SEM of three independent experiments performed in triplicates and are presented as relative light units (RLU) of fluorescence

intensity to firefly luciferase. **B** Non-linear regression model fitting of the Wnt activation curve in the presence of MFH-ND, using the formula:  $Y = X^h \times X_{Max}/(X^h + EC_{50}^h)$ . The curve fitting analysis was conducted with GraphPad Prism 9 v9.3.1, with h,  $X_{Max}$ , and EC<sub>50</sub> representing the three curve fitting parameters

10

Wnt3a (nM)

15

20



**Fig. 2** Hill factors determined by Hill plot analysis. Hill plots were generated to calculate Hill factors for **A** Wnt3a alone, **B** Wnt3a with 200 mM MFH-ND, and **C** Wnt3a with 400 mM MFH-ND. The Hill–Langmuir equation,  $\log[y(1-y)]$  versus  $\log [x]$  [18], was employed to construct the hill plot and obtain the hill coefficient. In the equation,

y represents Wnt activation measured in the TOP-Flash assay, while x corresponds to the concentration of Wnt3a. The slope of the plotted line represents the Hill coefficient, or Hill factor, and the standard error indicates the error in the coefficient estimation

of both LRP5 and LRP6, it is not as potent as Wnt3a in activating Wnt/ $\beta$ -catenin signaling in HEK293STF cells. Our experiments showed that the Wnt activity induced by 400  $\mu$ M MFH-ND in the cells is comparable to the activity induced by 2.7 nM Wnt3a. Therefore, to mimic a Wnt protein, we utilized two concentrations of MFH-ND: 200  $\mu$ M and 400  $\mu$ M, respectively.

We observed that MFH-ND alone demonstrated limited ability to activate Wnt signaling in the cells at the tested concentration. However, when combined with Wnt3a, MFH-ND significantly enhanced Wnt3a-induced Wnt activity, suggesting a synergistic effect between the two. Remarkably, the combined effect of Wnt3a and MFH-ND surpassed the sum of their individual effects at equivalent concentrations. Curve fitting analysis revealed that Wnt3a alone induced a maximum Wnt activity of  $1.1 \pm 0.1 \times 10^5$ . In contrast, the presence of 200 µM and 400 µM MFH-ND with Wnt3a resulted in maximum Wnt activities of  $2.7 \pm 0.8 \times 10^5$  and  $2.8 \pm 0.2 \times 10^5$ , respectively (Fig. 1B). Furthermore, our observations exhibited Hill factors exceeding 1.0 when fitting the curves of Wnt3a-induced Wnt activity at both MFH-ND concentrations. The calculated Hill factor was  $2.2 \pm 0.5$  in the presence of 200  $\mu$ M MFH-ND and 3.0  $\pm$  0.5 in the presence of 400 µM MFH-ND.

To further confirm the accuracy of the calculated Hill factors obtained through curve fitting, we conducted a Hill plot analysis [18] for all three conditions: Wnt3a alone, Wnt3a with 200  $\mu$ M MFH-ND, and Wnt3a with 400  $\mu$ M MFH-ND (Fig. 2). Our analysis revealed Hill factors of  $3.5 \pm 0.5$ ,  $2.2 \pm 0.5$ , and  $3.0 \pm 0.5$ , respectively, which aligns with the results obtained from curve fitting. These findings provide additional evidence that the synergistic effect of Wnt3a and MFH-ND on Wnt signaling induction is a positively cooperative event.

#### Discussion

According to the concept that Wnt proteins interact with Wnt receptors, leading to the formation of Wnt signalosomes at the cell membrane [19, 20], our findings support the notion that the initiation of Wnt3a-induced Wnt signalosomes follows a positively cooperative mechanism. Particularly, the effect of Wnt signalosomes is most pronounced during the early stages of their formation. In our study, we observed a 27-fold increase in Wnt activity inside the cells when the concentration of Wnt3a was doubled, indicating that a minimum concentration of 5.3 nM Wnt3a is sufficient to induce Wnt signalosome formation (Fig. 3). However, due to the positive cooperativity of the process, we further observed an additional 8.5-fold enhancement in cellular Wnt activity when the Wnt3a concentration was doubled from 5.3 to 10.7 nM. To address the possible synergistic interactions between different Wnt proteins [10, 11], we utilized the molecule MFH-ND as a mimic of another Wnt ligand [17]. While MFH-ND alone demonstrated low potency in activating Wnt signaling in the Top-Flash assay, we observed that at a concentration of 400 µM, it induced similar Wnt activity as Wnt3a at 2.7 nM. This implies that 400 µM MFH-ND possesses equivalent potency to another Wnt protein at 2.7 nM. Remarkably, when combining 2.7 nM Wnt3a with 200 µM MFH-ND, we observed significantly higher Wnt activity (41-fold) compared to Wnt3a alone at 5.3 nM (27fold). Furthermore, the augmented Wnt activity induced by the MFH-ND and Wnt3a combination, relative to the equivalent concentration of Wnt3a alone, remained consistent across different concentrations. For instance, the combination of 5.3 nM Wnt3a and 400 µM MFH-ND resulted in a 389-fold increase in Wnt activation, whereas 8 nM of Wnt3a alone induced only 124-fold Wnt activity (Fig. 3).

Based on the concept of Wnt signalosome [8], the study suggests that multiple canonical Wnt proteins can synergistically work together to form larger signalosomes on the cell

**Fig. 3** Binding capacity of MFH-ND expressed as fold change (Wnt3a at 2.7 nM). Graphical representation of luciferase assay showing the binding capacity of Wnt3a, MFH-ND, and the combination of Wnt3A + MFH-ND at increasing concentrations





Fig.4 Working model illustrating the dynamic process of signalosome assembly triggered by multiple Wnt proteins. The model shows the synergistic effect of multiple canonical Wnt proteins, leading to

membrane, resulting in stronger intracellular Wnt signaling (Fig. 4). This phenomenon is likely due to the binding of multiple different Wnt proteins to different FZD receptors. Consequently, the presence of different Wnt proteins within a Wnt signalosome brings additional FZD receptors to the signalosome, creating a larger signalosome (Fig. 4).

Therefore, it is possible that the concentration gradient of canonical Wnt proteins plays a crucial role in controlling the size and stability of the Wnt signalosome. Higher concentrations of canonical Wnt proteins promote the formation of larger signalosomes and increase their stability. Thus, higher concentrations not only induce higher Wnt activity but also prolong the duration of the induced Wnt activity.

In summary, our study illuminates the robust morphogenic properties associated with canonical Wnt proteins [1], given their pivotal role in regulating stem cell renewal, tissue homeostasis, and tissue repair [1]. These effects are underpinned by the positive cooperation observed during Wnt signalosome formation. In the context of cancer, the formation of an irregular Wnt signalosome can contribute to the stabilization of aberrant signaling due to formation of large Wnt signalosomes. Therefore, our finding in the current study underscores the challenges involved in developing therapeutic inhibitors that specifically target abnormal Wnt signaling pathways in cancer [21].

### **Materials and methods**

To carry out cell-based Wnt luciferase reporter assay, stably transfected HEK293 luciferase-based reporter cell line, HEK293STF (CRL-3249, ATCC, Manassas, VA), the formation of larger signalosomes on the cell membrane. This results in enhanced intracellular Wnt signaling, potentially attributed to the binding of different Wnt proteins to various FZD receptors

which expresses firefly luciferase under the control of T-cell factor/lymphoid enhancer factor (TCF/LEF) promoter [15], were used to examine the activation of the small molecule MFH-ND on activating the canonical Wnt signaling pathway. HEK293STF cells were cultured in 5% CO<sub>2</sub> at 37 °C in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L D-glucose and 2 mM glutamine (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen), 0.1 mM nonessential amino acids (Gibco), and 10 mM HEPES (Gibco). The cells were seeded at  $2 \times 10^5$  cells/well in a 96-well plate (Corning) and incubated overnight, and then were treated with vehicle (0.1% dimethyl sulfoxide (DMSO); Sigma-Aldrich), 0-18.7 nM recombinant human Wnt3a protein with carrier (5036-WN-010, R&D, Minneapolis, MN), 0-400 µM of MFH-ND, or Wnt3a (0-18.7 nM) with MFH-ND (0-400 µM), for 18 h. Cell viability and firefly luciferase activity were measured using the ONE-Glo<sup>™</sup> + Tox Luciferase Reporter and Cell Viability Assay kit (#E7120, Promega, Madison, WI). Microplate Reader, FilterMax F5 (Molecular Devices, Sunnyvale, California) was used to measure cell viability and firefly luciferase activity. The Wnt pathway activity was expressed as the relative light unit (RLU) of fluorescence intensity to firefly luciferase. Three individual experiments were performed in triplicate.

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Author contributions CB: experimental design, data acquisition, data analysis, manuscript draft and revisions, figure preparation; CSH: data acquisition, data analysis, manuscript revision, figure preparation; SXD and JJZ: conceptualization, experimental design, manuscript and figure revisions, final approval, funding acquisition, supervision.

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**Data availability** No datasets were generated or analysed during the current study.

#### Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Not applicable.

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