Inhibition by Chondroitin Sulfate E Can Specify Functional Wnt/β-Catenin Signaling Thresholds in NIH3T3 Fibroblasts

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Background: Wnt/β-catenin signaling is a therapeutic target in cancer and other diseases but also has important physiological functions.

Results: CS-E can inhibit Wnt/β-catenin signaling and interfere with selective transcriptional and biological outcomes.

Conclusion: Wnt/β-catenin signaling thresholds control distinct downstream transcriptional and biological outcomes.

Significance: The development of pharmacological strategies to dissociate disease-related from physiological functions of Wnt/β-catenin signaling is critical for clinical applications.

Aberrant activation of the Wnt/β-catenin signaling pathway is frequently associated with human disease, including cancer, and thus represents a key therapeutic target. However, Wnt/β-catenin signaling also plays critical roles in many aspects of normal adult tissue homeostasis. The identification of mechanisms and strategies to selectively inhibit the disease-related functions of Wnt signaling, while preserving normal physiological functions, is in its infancy. Here, we report the identification of exogenous chondroitin sulfate-E (CS-E) as an inhibitor of specific molecular and biological outcomes of Wnt3a signaling in NIH3T3 fibroblasts. We demonstrate that CS-E can decrease Wnt3a signaling through the negative regulation of LRP6 receptor activation. However, this inhibitory effect of CS-E only affected Wnt3a-mediated induction, but not repression, of target gene expression. We went on to identify a critical Wnt3a signaling threshold that differentially affects target gene induction versus repression. This signaling threshold also controlled the effects of Wnt3a on proliferation and serum starvation-induced apoptosis. Limiting Wnt3a signaling to this critical threshold, either by CS-E treatment or by ligand dilution, interfered with Wnt3a-mediated stimulation of proliferation but did not impair Wnt3a-mediated reduction of serum starvation-induced apoptosis. Treatment with pharmacological inhibitors demonstrated that both induction and repression of Wnt3a target genes in NIH3T3 cells require the canonical Wnt/β-catenin signaling cascade. Our data establish the feasibility of selective inhibition of Wnt/β-catenin transcriptional programs and biological outcomes through the exploitation of intrinsic signaling thresholds.

Wnt proteins are secreted glycoproteins that activate signaling pathways controlling a variety of developmental processes, including cell fate determination, morphogenesis, and differentiation, as well as tissue homeostasis (1–7). Aberrantly activated Wnt/β-catenin signaling has also been implicated in a number of human diseases and malignancies, including cancer, fibrosis, and cardiovascular disease (8–12). The involvement of activated Wnt/β-catenin signaling in human disease has sparked intense interest in the identification and characterization of inhibitory molecules that could be utilized to interfere with aberrant Wnt signaling (3, 13). However, although pharmacological inhibition of activated Wnt/β-catenin signaling is desirable for the treatment of many cancers, concomitant impairment of Wnt/β-catenin function in normal tissue physiology and homeostasis, for example in intestinal epithelium and liver, could have negative consequences (2, 14, 15). Thus, the ability to target disease-related biological outcomes of Wnt signaling while preserving the outcomes necessary for normal physiology would be critical for future clinical applications.

Several studies have demonstrated a correlation of Wnt/β-catenin signaling levels with embryonic stem cell differentiation, anterior specification during mouse embryogenesis, adult hepatic homeostasis, phenotypic severity of intestinal tumorigenesis, and lineage determination during hematopoiesis (16–21). However, it is not known whether Wnt signaling thresholds play a role in dissociating global Wnt transcriptional programs or the associated biological outcomes of Wnt/β-catenin signaling.

The glycosaminoglycan chondroitin sulfate (CS)2 consists of linear chains of repeating disaccharide units covalently linked to cell surface and secreted proteins to form chondroitin sulfate proteoglycans (22, 23), which have been shown to control multiple aspects of cellular behavior and communication (22, 24–27). Differentially sulfated CS forms include the monosulfated chondroitin 4-sulfate (C4S) and chondroitin 6-sulfate (C6S) units, as well as the disulfated units chondroitin sulfate D

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2 The abbreviations used are: CS, chondroitin sulfate; RP, recombinant protein; qRT-PCR, quantitative real-time RT-PCR; DMSO, dimethyl sulfoxide; EdU, 5-ethyl-2'-deoxyuridine; TCF/LEF, T-cell factor/lymphoid enhancer factor.
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(CS-D) and chondroitin sulfate E (CS-E). Differential sulfation of chondroitin can drastically affect its function (22, 28–31). Interestingly, exogenous CS-E has been shown to bind Wnt3a ligand with high affinity in vitro, and treatment of L-cells with CS-E, or alterations in endogenous levels of CS-E by modulations of expression of chondroitin-4-sulfotransferase-1 (C4ST-1), could affect the overall cellular levels of β-catenin (32, 33).

Here, we initially set out to determine the effects of exogenous CS-E treatment on Wnt3a signaling output in order to establish CS-E as a bona fide Wnt pathway inhibitor. We demonstrate that treatment with CS-E decreased Wnt3a signaling output by ~75%. Accordingly, treatment with CS-E was able to interfere with the phosphorylation of cell surface LRPs, a component of the Wnt receptor complex, suggesting that CS-E treatment interferes with Wnt3a-mediated receptor activation. Surprisingly, genome-wide gene expression profiling experiments demonstrated an inhibitory effect of CS-E on Wnt3a-mediated induction of target gene expression but not target gene repression. We go on to show that these effects of CS-E were due to differential requirements of Wnt3a signaling thresholds for induced versus repressed target genes and that different signaling thresholds also control specific biological outcomes of Wnt3a signaling. Consistent with our observations, CS-E is able to impair the Wnt3a-mediated stimulation of proliferation but cannot interfere with Wnt3a-mediated reduction of apoptosis. By utilizing Wnt3a ligand dilutions in the absence of CS-E, we demonstrate that the identified signaling threshold levels are an intrinsic property of the Wnt3a signaling cascade. Treatment with pharmacological inhibitors established that both induction and repression of Wnt3a target genes in NIH3T3 cells are mediated by the canonical Wnt/β-catenin signaling cascade. Thus, our data establish the feasibility of selective inhibition of Wnt3a transcriptional programs and biological outcomes through the exploitation of intrinsic signaling thresholds. We believe that these data will have an important impact on future functional evaluations of differential biological impacts of Wnt pathway inhibitors. This work is of substantial clinical interest, as it is a first step toward a better understanding of pathway inhibitors that interfere with specific disease-related outcomes of Wnt signaling while sparing physiological functions. Moreover, we provide support for a potential use of CS-E as a therapeutic inhibitor of specific biological outcomes of canonical Wnt signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Reagents, and Treatments**—NIH3T3, L-cells, and L-Wnt3a-cells (34) were obtained from ATCC. C45, C65, CS-D, and CS-E were obtained from Seikagaku/The Associates of Cape Cod. Wnt3a recombinant protein (Wnt3a-RP) was purchased from R&D Systems. Wnt antagonist I (WIR-1-endo; catalogue No. 681669) was purchased from Calbiochem and quercetin hydrate from Acros Organics (catalogue No. 681669) was purchased from Calbiochem and quercetin hydrate from Acros Organics (catalogue No. 681669). Wnt3a recombinant protein (Wnt3a-RP) was purchased from Calbiochem and quercetin hydrate from Acros Organics (catalogue No. 681669). Wnt3a ligand with high affinity in vitro, and treatment of L-cells with CS-E, or alterations in endogenous levels of CS-E by modulations of expression of chondroitin-4-sulfotransferase-1 (C4ST-1), could affect the overall cellular levels of β-catenin (32, 33).

Next, we set out to determine the effects of exogenous CS-E treatment on Wnt3a signaling output in order to establish CS-E as a bona fide Wnt pathway inhibitor. We demonstrate that treatment with CS-E decreased Wnt3a signaling output by ~75%. Accordingly, treatment with CS-E was able to interfere with the phosphorylation of cell surface LRPs, a component of the Wnt receptor complex, suggesting that CS-E treatment interferes with Wnt3a-mediated receptor activation. Surprisingly, genome-wide gene expression profiling experiments demonstrated an inhibitory effect of CS-E on Wnt3a-mediated induction of target gene expression but not target gene repression. We go on to show that these effects of CS-E were due to differential requirements of Wnt3a signaling thresholds for induced versus repressed target genes and that different signaling thresholds also control specific biological outcomes of Wnt3a signaling. Consistent with our observations, CS-E is able to impair the Wnt3a-mediated stimulation of proliferation but cannot interfere with Wnt3a-mediated reduction of apoptosis. By utilizing Wnt3a ligand dilutions in the absence of CS-E, we demonstrate that the identified signaling threshold levels are an intrinsic property of the Wnt3a signaling cascade. Treatment with pharmacological inhibitors established that both induction and repression of Wnt3a target genes in NIH3T3 cells are mediated by the canonical Wnt/β-catenin signaling cascade. Thus, our data establish the feasibility of selective inhibition of Wnt3a transcriptional programs and biological outcomes through the exploitation of intrinsic signaling thresholds. We believe that these data will have an important impact on future functional evaluations of differential biological impacts of Wnt pathway inhibitors. This work is of substantial clinical interest, as it is a first step toward a better understanding of pathway inhibitors that interfere with specific disease-related outcomes of Wnt signaling while sparing physiological functions. Moreover, we provide support for a potential use of CS-E as a therapeutic inhibitor of specific biological outcomes of canonical Wnt signaling.
Western Blots—NIH3T3 cells were stimulated with L-CM or Wnt3a-CM for 1 h in the presence or absence of CS-E at 100 μg/ml. Alternatively, NIH3T3 cells were stimulated with Wnt3a-RP (500 ng/ml) in the presence or absence of CS-E at 200 μg/ml. Whole cell lysates were separated on 6% SDS-polyacrylamide gels. Antibodies used included rabbit anti-LRP6, rabbit anti-pLRP6 (both from Cell Signaling Inc.), and mouse anti-α-tubulin (Santa Cruz Biotechnology).

RESULTS

CS-E Interferes with Wnt3a Signaling—In initial experiments, we set out to evaluate whether treatment with exogenous CS could interfere with Wnt3a signaling output. We stimulated canonical Wnt signaling in NIH3T3 cells with Wnt3a conditioned medium (Wnt3a-CM) from L-cells stably overexpressing a Wnt3a cDNA; and as a control, we used conditioned medium from the parental nontransfected L-cells (L-CM). To test the effects of differentially sulfated chondroitin forms on canonical Wnt signaling, we treated these cultures concomitantly with C4S, C6S, CS-D, or CS-E, all at 100 μg/ml, or with no CS as a control. Immunofluorescence staining for β-catenin showed that in L-CM-treated control cultures, low levels of β-catenin were located mainly in cell membrane and cytoplasmic regions (Fig. 1A, white arrow). Treatment with C4S (Fig. 1B), C6S (Fig. 1C), CS-D (Fig. 1D), or CS-E (Fig. 1E) did not alter β-catenin localization or levels. When control NIH3T3 cultures where treated with Wnt3a-CM, we observed a drastic accumulation and nuclear localization of β-catenin (Fig. 1F, yellow arrow) as shown previously. Concomitant treatment with C4S (Fig. 1G), C6S (Fig. 1H), or CS-D (Fig. 1I) did not alter this Wnt3a-mediated effect. However, CS-E was able to severely reduce the Wnt3a-mediated accumulation of β-catenin (Fig. 1J); but CS-E did not completely abolish the effect of Wnt3a, as weak nuclear staining for β-catenin was still visible (Fig. 1J, yellow arrow) indicating that residual Wnt3a signaling was still present. Quantification of β-catenin nuclear luminosity from Wnt3a-CM-treated cultures confirmed that treatment with C4S, C6S, and CS-D did not interfere with the Wnt3a-mediated nuclear accumulation of β-catenin (Fig. 1K). However, treatment with CS-E decreased the nuclear luminosity of β-catenin to ~27% (Fig. 1K).

We next examined whether CS-E could also interfere with the nuclear function of β-catenin. To this end, we employed the widely used TOPFLASH reporter assay, in which a luciferase reporter is driven by multiple T-cell factor/lymphoid enhancer factor binding sites and serves as a functional nuclear output of β-catenin activity. Stimulation with Wnt3a-CM led to a strong increase in TOPFLASH activity when compared with stimulation with L-CM (Fig. 1L). This was not altered by treatment with C4S, C6S, or CS-D; however, CS-E reduced Wnt3a-CM-stimulated TOPFLASH activity significantly (Fig. 1L). Quantitation of this effect showed that CS-E could reduce Wnt3a-CM-mediated TOPFLASH activity to ~20–25% (Fig. 1L), corroborating our quantitative findings above. Dose-response experiments established that CS-E at 5 μg/ml had a moderate effect on Wnt3a-CM-induced TOPFLASH activity that did not reach statistical significance, whereas CS-E at 20 μg/ml reduced TOPFLASH activity significantly, and CS-E at 100 μg/ml reduced TOPFLASH activity even further, with an apparent plateau reached at 100 μg/ml and no statistical difference between 20, 100, and 200 μg/ml (Fig. 1M). These data demonstrated that CS-E at 100 μg/ml achieved a maximum inhibitory effect on Wnt3a signaling when stimulating with Wnt3a-CM.

CS-E Interferes with LRP6 Phosphorylation—Previous Bia-core experiments showed that CS-E can bind Wnt3a ligand with high affinity (32). Thus, we hypothesized that CS-E treatment could interfere with the activation of the cell surface receptor complex by Wnt3a ligand. To this end, we analyzed phosphorylation of LRP6, an integral component of the Wnt receptor complex (Fig. 1N). Western blot analysis of NIH3T3 total cell lysates showed that treatment with Wnt3a-CM for 60 min led to phosphorylation of LRP6 (pLRP6), whereas concomitant treatment with CS-E abolished this phosphorylation (Fig. 1N). The levels of total LRP6 protein were not significantly altered by our treatments, and CS-E alone had no effect on pLRP6 levels. Quantitation of these effects showed a significant reduction in the levels of pLRP6 when cells were co-treated with Wnt3a-CM and CS-E, whereas CS-E alone did not alter pLRP6 levels (Fig. 1N). These results demonstrated that CS-E could interfere with Wnt3a-mediated activation of its cell surface receptor complex.

CS-E Interferes with Pathway Stimulation by Both Wnt3a-CM and Wnt3a-RP—To corroborate that this effect of CS-E on Wnt3a signaling is not caused by nonspecific interactions of CS-E with components of the conditioned medium other than Wnt3a, we also analyzed the effects of CS-E on β-catenin accumulation and localization, TOPFLASH activity, and LRP6 phosphorylation in response to recombinant Wnt3a protein (Wnt3a-RP) (supplemental Fig. S1). First, NIH3T3 cells were treated with Wnt3a-RP in the presence or absence of CS-E at 50, 100, 200, or 300 μg/ml. CS-E was able to significantly inhibit Wnt3a-RP-induced TOPFLASH activity at all concentrations, with a plateau effect at 200 μg/ml (Fig. supplemental S1A). We next analyzed the effect of CS-E treatment on Wnt3a-RP-stimulated LRP6 phosphorylation. Western blot analysis demonstrated that treatment with CS-E could significantly reduce Wnt3a-RP-mediated phosphorylation of LRP6 (supplemental Fig. S1B). Finally, we analyzed the effects of CS-E on Wnt3a-RP-stimulated accumulation and nuclear localization of β-catenin in immunofluorescence staining (supplemental Fig. S1, C–F). As shown for Wnt3a-CM above, CS-E treatment drastically reduced Wnt3a-RP-stimulated β-catenin staining (supplemental Fig. S1F) when compared with cultures treated with Wnt3a-RP alone (supplemental Fig. S1E). Measurement and quantification of β-catenin nuclear luminosity from supplemental Fig. S1, E and F showed that CS-E treatment reduced Wnt3a-RP-stimulated β-catenin staining to ~29% (supplemental Fig. S1G). Together, these data demonstrated that treatment with exogenous CS-E could interfere in a basically identical manner with signaling initiated by either Wnt3a-CM or Wnt3a-RP, thus corroborating our conclusions above that CS-E treatment negatively regulates Wnt3a signaling.

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CS-E Interferes with Wnt3a-mediated Induction, but Not Repression, of Target Genes—As a next step, we wanted to extend our analysis to the effects of CS-E treatment on Wnt3a-controlled global transcriptional programs. NIH3T3 cells were treated with L-CM or Wnt3a-CM in the presence or absence of exogenous CS forms at 100 μg/ml. Treatment with L-CM results in low levels of β-catenin expression, localized mainly in the cytoplasm and the cell membrane (A, white arrow). This pattern of β-catenin expression was identical in cultures treated with C4S (B), C6S (C), CS-D (D), and CS-E (E). Treatment with Wnt3a-CM caused a drastic increase in β-catenin levels and nuclear accumulation (F, yellow arrow). This outcome of Wnt3a-CM was unaffected by treatment with C4S (G), C6S (H), and CS-D (I). CS-E treatment caused a severe reduction in β-catenin expression levels (J) while still maintaining nuclear localization (F–J, yellow arrows). Scale bar = 10 μm. K, quantification of nuclear luminosity of Wnt3a-induced nuclear β-catenin signal from experiment depicted in F–J. Treatment with CS-E, but not C4S, C6S, or CS-D, reduced nuclear luminosity to ~27% when compared with Wnt3a treatment alone. L, treatment with Wnt3a-CM for 24 h caused a significant increase in TOPFLASH luciferase activity when compared with treatment with L-CM. Concomitant treatment with CS-E, but not C4S, C6S, or CS-D (all at 100 μg/ml), significantly reduced TOPFLASH reporter activity (*, p < 0.01). M, dose response of the effects of CS-E on Wnt3a-induced TOPFLASH activity. CS-E at 5 μg/ml reduced Wnt3a-induced TOPFLASH activity slightly, whereas CS-E at 20, 100, and 200 μg/ml reduced the effects of Wnt3a-CM stimulation significantly (*, p < 0.01). No significant differences among CS-E concentrations of 20, 100, and 200 μg/ml were observed. N, treatment of NIH3T3 cells with Wnt3a-CM (W3a) but not L-CM (L) for 1 h led to phosphorylation of LRP6 (pLRP6). Concomitant treatment with CS-E (E) (100 μg/ml) significantly (*, p < 0.05) interfered with phosphorylation of the LRP6 protein. Levels of LRP6 and α-tubulin proteins are shown as controls. Note that only the upper band of the two specific LRP6 bands is phosphorylated in response to Wnt3a.

CS-E profiles were analyzed by microarray analysis on Illumina Mouse BeadChips. We chose a time frame of 36 h to capture not only immediate early target genes but also a complete set of target genes affected by Wnt3a signaling. This analysis revealed 96 genes that were induced more than 2-fold by Wnt3a-CM treatment (supplemental Table S1), whereas 22

FIGURE 1. CS-E treatment interferes with Wnt3a signaling in NIH3T3 cells. A–J, immunofluorescence detection of β-catenin in NIH3T3 cells treated with L-CM or Wnt3a-CM in the presence or absence of exogenous CS forms at 100 μg/ml. Treatment with L-CM results in low levels of β-catenin expression, localized mainly in the cytoplasm and the cell membrane (A, white arrow). This pattern of β-catenin expression was identical in cultures treated with C4S (B), C6S (C), CS-D (D), and CS-E (E). Treatment with Wnt3a-CM caused a drastic increase in β-catenin levels and nuclear accumulation (F, yellow arrow). This outcome of Wnt3a-CM was unaffected by treatment with C4S (G), C6S (H), and CS-D (I). CS-E treatment caused a severe reduction in β-catenin expression levels (J) while still maintaining nuclear localization (F–J, yellow arrows). Scale bar = 10 μm. K, quantification of nuclear luminosity of Wnt3a-induced nuclear β-catenin signal from experiment depicted in F–J. Treatment with CS-E, but not C4S, C6S, or CS-D, reduced nuclear luminosity to ~27% when compared with Wnt3a treatment alone. L, treatment with Wnt3a-CM for 24 h caused a significant increase in TOPFLASH luciferase activity when compared with treatment with L-CM. Concomitant treatment with CS-E, but not C4S, C6S, or CS-D (all at 100 μg/ml), significantly reduced TOPFLASH reporter activity (*, p < 0.01). M, dose response of the effects of CS-E on Wnt3a-induced TOPFLASH activity. CS-E at 5 μg/ml reduced Wnt3a-induced TOPFLASH activity slightly, whereas CS-E at 20, 100, and 200 μg/ml reduced the effects of Wnt3a-CM stimulation significantly (*, p < 0.01). No significant differences among CS-E concentrations of 20, 100, and 200 μg/ml were observed. N, treatment of NIH3T3 cells with Wnt3a-CM (W3a) but not L-CM (L) for 1 h led to phosphorylation of LRP6 (pLRP6). Concomitant treatment with CS-E (E) (100 μg/ml) significantly (*, p < 0.05) interfered with phosphorylation of the LRP6 protein. Levels of LRP6 and α-tubulin proteins are shown as controls. Note that only the upper band of the two specific LRP6 bands is phosphorylated in response to Wnt3a.
these genes (supplemental Table S2 and Fig. 2, 2 genes in this group (supplemental Table S2 and Fig. 2). Wnt3a interferes with Wnt3a-mediated repression of any of the target genes induced 2-fold or more by Wnt3a-CM in NIH3T3 cells. Wnt3a-CM (W3a, marked in blue) increased mRNA expression of these target genes from −7- to 2-fold over treatment with L-CM. Concomitant treatment with CS-E at 100 μg/ml (W3a+CS-E, marked in red) reduced mRNA expression of all Wnt3a-induced genes. Treatment with CS-E alone in L-CM (CS-E, marked in yellow) did not significantly affect target gene expression over L-CM alone. B, quantification of results shown in A. In the presence of Wnt3a-CM, CS-E reduced target gene expression to −18%. CS-E did not have a significant effect on gene expression in L-CM alone. C, graphic presentation of the fold-mRNA expression of the 22 genes repressed 2-fold or more by Wnt3a-CM in NIH3T3 cells. Wnt3a-CM (blue) repressed mRNA expression of these target genes to levels of −0.2–0.5 over treatment with L-CM. Concomitant treatment with CS-E at 100 μg/ml (red) was not able to interfere with this repression and in many cases slightly increased repression of mRNA expression. Treatment with CS-E alone in L-CM (yellow) also slightly decreased target gene expression over L-CM alone. D, quantification of results shown in C. CS-E treatment could not interfere with Wnt3a-CM-mediated target gene repression and instead increased repression slightly. A similar effect was observed when cells were treated with CS-E in L-CM alone.

FIGURE 2. Microarray analysis: CS-E differentially affects positively and negatively regulated Wnt3a transcriptional programs. A, graphic presentation of the fold-change in mRNA expression of the 96 genes induced 2-fold or more by Wnt3a-CM in NIH3T3 cells. Wnt3a-CM (W3a, marked in blue) increased mRNA expression of these target genes from −7- to 2-fold over treatment with L-CM. Concomitant treatment with CS-E at 100 μg/ml (W3a+CS-E, marked in red) reduced mRNA expression of all Wnt3a-induced genes. Treatment with CS-E alone in L-CM (CS-E, marked in yellow) did not significantly affect target gene expression over L-CM alone. B, quantification of results shown in A. In the presence of Wnt3a-CM, CS-E reduced target gene expression to −18%. CS-E did not have a significant effect on gene expression in L-CM alone. C, graphic presentation of the fold-mRNA expression of the 22 genes repressed 2-fold or more by Wnt3a-CM in NIH3T3 cells. Wnt3a-CM (blue) repressed mRNA expression of these target genes to levels of −0.2–0.5 over treatment with L-CM. Concomitant treatment with CS-E at 100 μg/ml (red) was not able to interfere with this repression and in many cases slightly increased repression of mRNA expression. Treatment with CS-E alone in L-CM (yellow) also slightly decreased target gene expression over L-CM alone. D, quantification of results shown in C. CS-E treatment could not interfere with Wnt3a-CM-mediated target gene repression and instead increased repression slightly. A similar effect was observed when cells were treated with CS-E in L-CM alone.

genes were repressed more than 2-fold by Wnt3a-CM treatment (supplemental Table S2). Several genes in both the induced and repressed groups were identified previously as Wnt3a target genes in NIH3T3 cells after 6 h of treatment (supplemental Tables S1 and S2) (36). Identification of additional target genes suggested that our 36-h treatment might include direct target genes that require treatment with Wnt3a for more than 6 h and/or indirect target genes. When we examined the effects of CS-E treatment on Wnt3a-CM target gene induction, we observed a uniform reduction in the Wnt3a-induced expression of all positively regulated target genes (supplemental Table S1 and Fig. 2A). Quantification showed that on average, CS-E treatment reduced Wnt3a-CM-stimulated gene expression to −18%, when compared with Wnt3a treatment in the absence of CS-E (Fig. 2B). This result correlated with the CS-E-mediated reduction in Wnt3a signaling described above. Notably, CS-E alone did not significantly affect expression of these Wnt3a target genes (Fig. 2, A and B). We then analyzed the effect of CS-E on expression of target genes negatively regulated by Wnt3a. Surprisingly, treatment with CS-E did not interfere with Wnt3a-mediated repression of any of the target genes in this group (supplemental Table S2 and Fig. 2C). Quantification of this effect demonstrated no interference of CS-E with the negative regulation of Wnt3a of these target genes (Fig. 2D). CS-E alone had a modest overall repressive function on these genes (supplemental Table S2 and Fig. 2, C and D). Thus, these results suggested a surprising differential effect of CS-E on Wnt3a-mediated gene induction versus repression. To confirm these results, we selected five positively regulated Wnt target genes (calrecticulin (Calr), Rho GTPase-activating protein 18 (Arhgap18), Prickle homolog 1 (Prickle1), Tumor necrosis factor ligand superfamily 9 (Tnfsf9), MAS-related GPR, member F (Mrgprf)) and five negatively regulated target genes (cytochrome P450 polypeptide 2 (Cyp2f2), secretory leukocyte peptidase inhibitor (Slpi), arrestin domain-containing protein 4 (Arrdc4), lipocalin 2 (Lcn2), SRY-box-containing gene 9 (Sox9)) and quantified the effect of CS-E treatment on Wnt3a stimulation of target gene mRNA expression by qRT-PCR (Fig. 3). These experiments confirmed the ability of Wnt3a-CM to induce expression of all five positive target genes when compared with treatment with L-CM (Fig. 3A). Concomitant treatment with CS-E caused a significant reduction in the expression of these positively regulated target genes, (Fig. 3A), confirming that CS-E could interfere with the Wnt3a-mediated stimulation of target gene expression. All genes in the group of negatively regulated target genes were drastically repressed by Wnt3a-CM (Fig. 3B); importantly, these experiments confirmed that concomitant treatment with CS-E did not interfere with Wnt3a repression and therefore could not reverse the Wnt3a-mediated reduction in target gene expression (Fig. 3B). This effect was observed independently of the effect of CS-E alone on target gene expression (Fig. 3B), suggesting that the absence of any effect of CS-E on Wnt3a-mediated target gene repression was not due to strong independent effects of CS-E.
on expression of these genes. These results confirmed our microarray data and showed that CS-E could interfere with Wnt3a-mediated induction, but not repression, of target gene mRNA expression. Because we showed above that even in the presence of CS-E a residual Wnt3a signaling output of ~25% was maintained, we hypothesized that the differential effects of CS-E on Wnt3a target gene induction versus repression might have identified a critical Wnt3a signaling threshold that allows selective control of global Wnt3a transcriptional programs.
FIGURE 4. Differential Wnt3a thresholds control target gene induction versus repression. A and C, TOPFLASH reporter assays in NIH3T3 cells in the presence of Wnt3a-CM at 0 (noW), 10 (W10%), 25 (W25%), 50 (W50%), or 100% (W100%) led to a gradient of Wnt3a signaling activity with 0, 16, 30, 55, and 100% TOPFLASH activity, respectively. B, expression of the positively regulated Wnt3a target genes Mrgprf, Tnfsf9, Arhgap18, and Prickle1 at different Wnt3a signaling levels. Low levels of 8–18% induction were observed at Wnt3a signaling levels of 16% (W10%), whereas a 17–42% induction of expression was observed at a Wnt3a signaling level of 30% (W25%). Even at 55% signaling activity (W50%), expression levels of induced target genes only reached 10–38% of the levels observed at 100% signaling activity (W100%). D, the negatively regulated Wnt3a target genes Cyp2f2, Lcn2, Arrdc4, and Txnip showed repression of 35–75% even at the lowest signaling levels tested (16% = W10%). At 30% Wnt3a signaling activity (W30%), we observed 68–89% repression, and at 55% signaling activity (W50%) repression reached 40–94% of the levels observed at 100% signaling activity (W100%). The dashed line box indicates signaling levels that bracket the levels we observed with CS-E treatment (25%; see Fig. 1).
Differential Wnt3a Signaling Thresholds for Induced Versus Repressed Target Genes—To test our hypothesis that differential Wnt3a signaling thresholds govern target gene induction versus repression, and to ensure that the effects we observed were not due to nonspecific effects of CS-E, we modulated Wnt3a signaling levels in the absence of CS-E. We generated a Wnt3a signaling gradient by dilution of CM with 0, 16, 30, 55, and 100% TOPFLASH activity in conditions with 0, 10, 25, 50, and 100% Wnt3a-CM, respectively (Fig. 4, A and C). We then asked whether expression of positive and negative Wnt3a target genes in NIH3T3 cells was responsive to different Wnt3a signaling levels. Analysis of expression of the positively regulated target genes Mrgprf, Tuf59, Arhgap18, and Prickle1 by qRT-PCR showed low levels of ~8–18% induction at Wnt3a signaling levels of 16% (Fig. 4B, W100), whereas a 17–42% induction of expression was observed at a Wnt3a signaling level of 30% (W25%). Even at 55% signaling activity (Fig. 4B, W50%) expression levels of induced target genes reached only 10–38% of the levels observed at 100% signaling activity (W100%). In contrast, the negatively regulated Wnt3a target genes Cyp2j2, Lcn2, Arrdc4, and Tnxis showed 35–75% repression even at the lowest signaling levels tested (16%) (Fig. 4D, W10%). At 30% Wnt3a signaling activity (W25%), we observed 68–89% repression, and at 55% signaling activity (W50%) repression reached 40–94% of the levels observed at 100% signaling activity (W100%). These results support our hypothesis that differential Wnt3a signaling thresholds control target gene induction versus repression. Whereas only minimal induction of target gene expression was achieved at low levels of Wnt3a signaling activity, gene repression was much more sensitive at these low signaling levels and allowed significant repression. These results also supported our notion that inhibition of Wnt3a signaling to ~25% by treatment with CS-E could interfere with target gene induction but not with target gene repression.

Differential Wnt3a Signaling Thresholds Regulate Proliferation Versus Apoptosis—Wnt/β-catenin signaling has been shown in many studies to regulate proliferation positively through the induction of genes involved in the positive regulation of the cell cycle and proliferation (37, 38). An analysis of Gene Ontology (GO) Biological Processes of the positively regulated genes in our gene profiling experiments indeed showed an enrichment in genes involved in the positive regulation of cell proliferation (supplemental Table S1). In contrast, canonical Wnt signaling has also been shown to interfere with serum starvation-induced apoptosis (38–40), which has been correlated with the repression of pro-apoptotic genes (36, 39).
Accordingly, analysis of GO Biological Processes, as well as literature searches, of the genes repressed by Wnt3a in our gene profiling experiments demonstrated an enrichment of pro-apoptotic genes (supplemental Table S2). Because we had demonstrated that Wnt3a could regulate positive and negative target genes at different threshold signaling levels, we hypothesized that these Wnt3a thresholds might also affect proliferation and apoptosis in a similar fashion. To test our hypothesis, we first examined at which signaling levels Wnt3a could efficiently induce proliferation of NIH3T3 cells. For this, we established Wnt3a-RP signaling gradients in TOPFLASH assays; concentrations of Wnt3a-RP of 0, 50, 62.5, 100, 167, and 500 ng/ml resulted in TOPFLASH activities of 0, 9, 27, 46, 76, and 100%, respectively (Fig. 5A). We then measured proliferation of NIH3T3 cells at these Wnt3a signaling levels by EdU incorporation. Cells were treated with Wnt3a-RP for 24 h, with the nucleotide analog EdU added for the last 60 min followed by the analysis of detection and quantification of EdU by immunofluorescence methods as an output for proliferation (Fig. 5B). Wnt3a signaling levels of 9% (50 ng/ml Wnt3a-RP), 27% (62.5 ng/ml Wnt3a-RP), 46% (100 ng/ml Wnt3a-RP), and 76% (167 ng/ml Wnt3a-RP) increased EdU incorporation by 1–10% when compared with unstimulated cells (Fig. 5B). At 100% signaling activity (500 ng/ml Wnt3a-RP), a dramatic increase of EdU-positive cells to 28% was observed (Fig. 5B). These data suggested that high levels of Wnt3a signaling, approaching 100% activity, are required in NIH3T3 cells to elicit a maximum effect on cell proliferation.

Next, we analyzed the effects of the different Wnt3a signaling levels established in Fig. 5A on serum starvation-induced apoptosis by TUNEL assays (Fig. 5C). Cells were starved for 24 h in medium containing 0.3% serum and were treated concomitantly with Wnt3a-RP at the indicated concentrations. As a control, cells were incubated in 10% serum. After treatments, cells were processed for TUNEL staining, and the percentage of positively stained nuclei was quantified (Fig. 5C). Cells in 10% serum showed ~3% TUNEL-positive cells; this increased to 33% when cell were incubated in 0.3% serum, thus demonstrating that serum starvation could lead to increased apoptosis in these cells. A low Wnt3a signaling activity of 9% (50 ng/ml Wnt3a-RP) did not interfere with apoptosis, but signaling activity of 27% (62.5 ng/ml Wnt3a-RP) significantly reduced apoptosis to ~13%. Strikingly, higher Wnt3a signaling activities of 46, 76, or 100% did not increase this anti-apoptotic effect any further (Fig. 5C), suggesting that a Wnt3a signaling threshold of ~27% is sufficient to mediate the full anti-apoptotic effects of Wnt3a. In summary, these data supported our hypothesis that Wnt3a can regulate proliferation and apoptosis at different threshold levels. Wnt3a signaling activity of close to 100% was required to efficiently promote proliferation, whereas a signaling activity of only 27% was sufficient for a maximum anti-apoptotic effect.

**CS-E Treatment Can Interfere with Wnt3a-induced Proliferation but Not with Wnt3a-mediated Repression of Apoptosis**—The data given above led us to hypothesize that reduction of Wnt3a signaling to ~25% by treatment with CS-E will interfere with the effects of Wnt3a signaling on proliferation but will not affect the functional role of Wnt3a signaling in the prevention of apoptosis in NIH3T3 cells. To investigate proliferative responses, cells were incubated in DMEM, 10% FBS in the presence or absence of Wnt3a-RP for 24 h, either alone (Fig. 6, A and D) or in the presence or absence of CS-D (Fig. 6, B and E) or CS-E (Fig. 6, C and F), followed by EdU incorporation. The percentage of EdU-positive cells was quantified by co-staining with DAPI. Few EdU-positive cells were seen in control conditions (Fig. 6A), and similar numbers of cells were observed when treated with CS-D (Fig. 6B) or CS-E (Fig. 6C). Stimulation with Wnt3a-RP caused an increase in EdU-positive cells (Fig. 6D), which was not altered by concomitant treatment with CS-D (Fig. 6E). However, concomitant treatment with CS-E led to a reduction in the number of EdU-positive cells (Fig. 6F). Quantification of these data (Fig. 6G) showed that the proliferation rate of ~42% in control conditions was increased to 54% with the addition of Wnt3a-RP. Treatment with CS-D did not change the pro-proliferative effect of Wnt3a, whereas CS-E completely abolished the ability of Wnt3a to stimulate proliferation (Fig. 6G). To corroborate these findings, we also sought to determine the effects of CS-E on Wnt3a-mediated cell growth over a longer time frame. Equal numbers of NIH3T3 cells were incubated for 24 h in the presence or absence of Wnt3a-RP, with or without CS-D or CS-E (Fig. 6H), followed by quantification of cell numbers. Wnt3a-RP treatment resulted in a 28% increase in cell growth; this was not significantly altered by concomitant CS-D treatment. However, the addition of CS-E completely abolished Wnt3a-stimulated cell growth and in fact led to a 2% decrease in cell numbers (Fig. 6H).

We next analyzed the effect of treatment with CS-D and CS-E on the rescue of serum starvation-induced apoptosis by Wnt3a (Fig. 6, I–N). NIH3T3 cells were serum-starved in medium containing 0.3% serum, with Wnt3a-RP at 500 ng/ml in the presence or absence of CS-D or CS-E at 100 µg/ml, for 24 h. Subsequently, TUNEL staining was performed to visualize cells undergoing apoptosis. Cells incubated in 10% serum showed very little positive staining (Fig. 6I). Serum starvation led to a significant increase in TUNEL-positive cells (Fig. 6J), which was significantly reduced by treatment with Wnt3a-RP (Fig. 6K). Concomitant treatment with CS-D did not alter this effect of Wnt3a (Fig. 6L). Importantly, treatment with CS-E also did not interfere with Wnt3a function in this assay (Fig. 6M). Quantification of these data (Fig. 6N) showed that almost no TUNEL-positive cells were present in 10% serum conditions, which increase to 47% TUNEL-positive cells in 0.3% serum. Treatment with Wnt3a-RP reduced the number of cells undergoing apoptosis to 12%, which was not significantly altered by CS-D (as we showed that CS-D did not alter Wnt3a signaling) or by CS-E (Fig. 6N). In summary, these data supported our hypothesis that a reduction of Wnt3a signaling levels to ~25% by treatment with exogenous CS-E interfered with Wnt3a-stimulated proliferation but not with the anti-apoptotic effects of Wnt3a in NIH3T3 cells.

**Wnt3a Signaling Thresholds Achieved by Pharmacological Inhibition Differentially Affect Induced Versus Repressed Target Genes**—Thus far, we have described novel Wnt3a signaling thresholds that differentially control target gene induction and repression as well as the biological consequences of a Wnt3a signal. We hypothesized that establishment of Wnt3a signaling
thresholds by treatment with previously established pharmacological inhibitors should differentially affect Wnt3a-mediated target gene expression in a manner similar to inhibition with CS-E or to dilution of Wnt3a ligand in the absence of any inhibitors. For this purpose, we utilized two known pharmacological inhibitors of the Wnt/β-catenin pathway: the tankyrase inhibitor IWR-1 (inhibitor of Wnt response-1), which stabilizes axin, a scaffold for the intracellular β-catenin destruction complex, and therefore inhibits canonical β-catenin signaling (41), and the flavonoid quercetin, which has been utilized to interfere with the transcriptional activity of β-catenin/TCF complexes in the nucleus (42, 43). We first utilized different concentrations of IWR-1 and quercetin in TOPFLASH assays to establish a Wnt3a signaling gradient (Fig. 7A). NIH3T3 cells were treated with DMSO (control) and IWR-1 (5, 10, 25, and 50 μM) and quercetin (50, 100, 150, and 500 μM) concomitantly with L-CM or Wnt3a-CM for 24 h. IWR-1 and quercetin inhibited Wnt3a signaling activity in a concentration-dependent manner. Importantly, IWR-1 at 5 μM and quercetin at 150 μM reduced Wnt3a/β-catenin-induced TOPFLASH...
cells. The presence or absence of IWR-1 and quercetin at 5 and 150 μM, respectively, followed by RNA preparation and qRT-PCR (Fig. 7B).

The expression levels of three positively regulated (Tnfsf9, Mrgprf, and Prickle1) and three negatively regulated (Lcn2, Casp12, and Arrdc4) Wnt3a target genes were analyzed. These experiments demonstrated that IWR-1 and quercetin, at concentrations that reduced Wnt/β-catenin signaling activity to ~25%, significantly reduced Wnt3a-mediated gene induction but could not interfere with Wnt3a-mediated target gene repression. Overall, these studies confirmed that a critical intrinsic Wnt3a signaling threshold, established by either Wnt3a ligand dilution or by pharmacological inhibition using CS-E, IWR-1, or quercetin, leads to differential effects on target gene induction versus repression.

Both Induction and Repression of Target Genes by Wnt3a Require the Canonical β-Catenin Pathway—It was reported recently that in articular chondrocytes, Wnt3a could simultaneously activate the canonical β-catenin and the noncanonical calcium/calmodulin kinase II (Ca2+/CaMKII) pathways (44). Therefore, we sought to determine whether target gene induction and repression by Wnt3a in our system was solely dependent on the canonical β-catenin pathway. For this purpose, we again utilized the pharmacological inhibitors IWR-1 and quercetin. We hypothesized that if either induction or repression of target genes was dependent on a noncanonical pathway, a complete inhibition of canonical β-catenin signaling by IWR-1 or quercetin should leave the noncanonical transcriptional response intact. We first established that IWR-1 at 100 μM and quercetin at 500 μM completely eliminated Wnt3a-mediated canonical β-catenin signaling in TOPFLASH assays (Fig. 8A). We then asked how this complete inhibition of β-catenin signaling by IWR-1 and quercetin would affect target gene induction and repression by Wnt3a. NIH3T3 cells were treated with Wnt3a-RP for 36 h in the presence or absence of IWR-1 at 100 μM or quercetin (500 μM). Subsequently, we prepared RNA and analyzed the effects of IWR-1 and quercetin on the expression levels of three positively regulated (Tnfsf9, Mrgprf, and Prickle1) and three negatively regulated (Lcn2, Casp12, and Arrdc4) Wnt3a target genes (Fig. 8B). Inhibition of canonical β-catenin signaling by IWR-1 and quercetin completely eliminated both the induction and repression of target genes by Wnt3a-RP (Fig. 8B), suggesting that both Wnt3a-induced and -repressed transcriptional programs require a functional canonical β-catenin pathway.

**DISCUSSION**

Here, we have demonstrated that the glycosaminoglycan CS-E can function as an inhibitor of Wnt/β-catenin signaling by interfering with Wnt3a-mediated phosphorylation of LRP6 and activation of the Wnt receptor complex. Surprisingly, genome-wide gene expression profiling experiments showed a selective inhibitory effect of CS-E on Wnt3a-mediated gene induction but not target gene repression. These effects of CS-E were due to differential Wnt3a signaling thresholds for induced versus repressed target genes. Moreover, these signaling thresholds also control the effects of Wnt3a on proliferation and apoptosis. Specifically, at a critical threshold level of ~25% signaling activity, Wnt3a could not efficiently induce target gene expression or stimulate cell proliferation. In contrast, 25% Wnt3a signaling activity was sufficient to efficiently repress activity to the critical threshold levels of 27 and 29%, respectively.

We then analyzed the effects of these inhibitor concentrations on Wnt3a-mediated target gene expression. NIH3T3 cells were treated with L-CM or Wnt3a-CM for 36 h in the presence or absence of IWR-1 and quercetin at 5 and 150 μM, respectively, followed by RNA preparation and qRT-PCR (Fig. 7B).
negatively regulated target genes and interfere with serum starvation-induced apoptosis. Consistent with these observations, CS-E, which reduced Wnt3a signaling to ~25%, was able to impair Wnt3a-mediated stimulation of proliferation but could not interfere with Wnt3a-mediated reduction of apoptosis (summarized in Fig. 9). These biologically important signaling thresholds were also observed when utilizing Wnt3a ligand dilutions in the absence of CS-E, thus demonstrating that the identified signaling thresholds are an intrinsic property of the Wnt3a signaling cascade. We also demonstrated that both positively and negatively regulated transcriptional programs downstream of Wnt3a required a functional canonical β-catenin signaling cascade. Of note, one negatively regulated target gene, *Txnip*, consistently showed stronger repression at

FIGURE 8. Induction and repression of Wnt3a target genes require the canonical β-catenin pathway. A, TOPFLASH assay to establish complete inhibition of Wnt/β-catenin signaling with IWR-1 and quercetin. NIH3T3 cells were treated for 24 h with DMSO (Control), IWR-1 at 100 μM, or quercetin at 500 μM either in control medium (DMEM/10% FBS) or in DMEM/10% FBS plus 500 ng/ml Wnt3a-RP. IWR-1 and quercetin at these concentrations completely blocked Wnt3a/β-catenin-induced TOPFLASH activity in NIH3T3 cells. B, NIH3T3 cells were treated with Wnt3a-RP (500 ng/ml) for 36 h in the presence or absence of IWR-1 and quercetin at 100 and 500 μM, respectively, followed by RNA preparation and qRT-PCR. The expression levels of three positively regulated (*Tnfsf9, Mrgprf, and Prickle1*) and three negatively regulated (*Lcn2, Casp12, and Arrdc4*) Wnt3a target genes were analyzed as described in the legend for Fig. 3. Complete inhibition of Wnt/β-catenin signaling eliminated both the induction and repression of target genes by Wnt3a-RP (*, p < 0.05).
efficiently or to promote proliferation. In contrast, target gene
but maintain aspects of Wnt/β-catenin to a specific signaling threshold might interfere with
disease-related biological outcomes, for example proliferation, apoptosis would still be possible at these positions in the gradient. Even further along the gradient, at very low signaling levels of ~9% and below, Wnt3a-mediated target gene repression and interference with apoptosis would also diminish.

We have shown here that the canonical β-catenin-dependent signaling cascade is critical for both positive and negative regulation of target gene expression by Wnt3a. The mechanism by which Wnt3a/β-catenin signaling differentially regulates transcriptional programs and biological function remains to be elucidated. In the absence of Wnt3a signaling, LEF and TCF function as transcriptional repressors by recruiting co-repressors such as Groucho to the classic Wnt response element (WRE) (45–47). When Wnt3a signaling is initiated, stabilized β-catenin can translocate to the nucleus and displace Groucho from the TCF/LEF complex, which switches the activity of the complex from a repressor to a transcriptional activator (47). However, recently published evidence suggests that β-catenin/TCF/LEF could also function as a transcriptional repressor through the binding of an alternative DNA regulatory sequence (48). In the context of our data, this could suggest that the interactions of a β-catenin/TCF/LEF protein complex with the classic Wnt response element to stimulate target gene expression might exhibit different kinetics from the interaction of the β-catenin/TCF/LEF complex with an alternative repressor DNA element.

A recent publication demonstrates that Wnt3a stimulation of articular chondrocytes could simultaneously activate β-catenin-dependent as well as Ca2+/CaMKII-dependent signaling pathways (44). These two pathways were activated at different Wnt3a signaling thresholds; at high concentrations, Wnt3a activates the β-catenin pathway, whereas lower signaling levels are sufficient to activate the Ca2+/CaMKII-dependent pathway (44). In contrast, our data presented here suggest that in NIH3T3 cells, both target gene induction and repression require a functional β-catenin cascade. It is intriguing to speculate that Wnt3a stimulation of different downstream pathways might be coordinated and regulated in a cell type- or context-dependent manner.

This work has important pharmacological implications. Wnt/β-catenin signaling is a critical therapeutic target in a number of human diseases including cancer, fibrosis, heart disease, and osteoarthritis (11, 49, 50). Thus, much effort has gone into the identification and characterization of Wnt pathway inhibitors, although translation of this research into clinical applications has not been successful thus far. Moreover, targeting Wnt signaling harbors the risk of interfering with the normal physiological functions of Wnt/β-catenin signaling (2, 14, 15). A detailed understanding of the effects of Wnt inhibitors on downstream disease-related versus physiological biological events is crucial for any translational efforts. We show here that CS-E could reduce Wnt3a signaling and interfere with target gene induction and proliferative responses but not with Wnt3a-mediated target gene repression and anti-apoptotic responses. Moreover, we demonstrate that the pharmacological Wnt inhibitors IWR-1 and quercetin also differentially affect target gene expression when utilized at concentrations that specify the critical canonical Wnt signaling threshold. Indeed, Shi et al.
(51) report that inhibition of Wnt/β-catenin signaling impairs proliferation and expression of MYC and CCND1, genes associated with proliferation, but did not affect apoptosis or expression of Survivin, an apoptosis-related gene. It remains to be seen whether the critical Wnt signaling threshold that we identified here is functional in different cell types or whether it is modulated in a cell type-specific or temporal manner in development and disease. Our work represents an important step toward targeting individual biological outcomes of Wnt signaling, for example increased proliferation in cancer progression, while leaving other outcomes, for example tissue homeostasis and repair, unaffected.

CS-E has been shown to interfere with tissue colonization of mouse osteosarcoma and lung cancer cells injected into the tail veins of recipient mice (52, 53). Our preliminary data indicate that CS-E could also interfere with breast cancer progression.3 We are currently investigating whether localized or systemic treatment with CS-E in clinically relevant mouse cancer models could indeed differentially affect specific downstream biological consequences of Wnt signaling and whether CS-E treatment is a promising therapeutic avenue in these models. From a clinical point of view, it is interesting to note that oral administration of CS, registered as a drug on the European market for the treatment of osteoarthritis symptoms, has been studied a clinical point of view, it is interesting to note that oral administration of CS-E, treatment with CS-E in clinically relevant mouse cancer models (54, 55).

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