

RESEARCH LETTER

Caffeine Limits
Expansion of *Apc*-
Deficient Clones
in the Intestine by
NOTUM Inhibition

Colorectal cancer (CRC) development is characterized by stepwise accumulation of mutations, of which the majority display early mutations in tumor suppressor gene *APC*.¹ Previous work revealed that *Apc* loss confers a competitive advantage to mutant intestinal stem cells (ISCs), which consequently replace all normal ISCs and drive crypt fixation in vivo.² Recent studies demonstrate that this advantage can be attributed to the secretion of Wnt antagonists (eg, NOTUM/WIF1/DKK2) that drive normal ISC differentiation.^{3,4} In particular, NOTUM, which functions as negative regulator of Wnt activity by deacylating Wnt ligands,⁵ poses an interesting chemoprevention target because it is highly up-regulated in *Apc*-mutant murine and human cells.^{3,4,6} Interestingly, recent work has identified caffeine as a potent NOTUM inhibitor by binding its catalytic pocket and thereby inhibiting its function.⁷ Therefore, in this study, we investigate the chemopreventive effects of caffeine on the expansion of *Apc*-mutant clones in the intestine (see also [Supplementary Methods](#)).

To assess the role of caffeine on Wnt signaling in vitro, we first validated the recently described inhibitory effect of caffeine on NOTUM⁷ by using a Wnt reporter cell line ([Supplementary Figure 1A and B](#)). Administration of recombinant NOTUM decreased Wnt pathway activity, an effect that is alleviated by supplementing 200 $\mu\text{mol/L}$ caffeine ([Supplementary Figure 1B](#)). Next, we investigated the effect of caffeine on intestinal organoids. We previously demonstrated that incubation of wild-type organoids with conditioned medium (CM) of *Apc*^{-/-} organoids resulted in loss of clonogenic potential.^{3,4}

Moreover, we demonstrated that dilution of CM results in a dose-dependent rescue of clonogenicity, an effect that occurred at lower dilution using CM derived from *Apc*^{-/-};*Notum*^{KO} organoids,³ highlighting the importance of NOTUM in executing the inhibitory effect.³ Here, we reveal that addition of 200 $\mu\text{mol/L}$ caffeine to *Apc*^{-/-} CM improves wild-type clonogenicity to a similar extent as previously reported using *Apc*^{-/-};*Notum*^{KO} CM³ ([Figure 1A](#), [Supplementary Figure 1C and D](#)). Importantly, no effect was observed between organoids incubated with *Apc*^{-/-};*Notum*^{KO} CM with or without caffeine ([Figure 1A](#), [Supplementary Figure 1E](#)). Together, these data indicate that caffeine reduces the Wnt inhibiting effects of *Apc*-mutant cells on their wild-type counterparts. Of note, caffeine treatment alone did not affect wild-type organoid growth, size, clonogenicity, and expression of Wnt target gene *Axin2* ([Supplementary Figure 2A–D](#)).

We next assessed the influence of caffeine on normal and mutant ISC dynamics in vivo. We induced *Apc* loss and traced the expansion of *Apc*-deficient clones over time in the absence or presence of caffeine administered in the drinking water (400 mg/L) ([Figure 1B](#)). As previously reported, *Apc*-mutant clones can be visualized by *Notum* expression ([Figure 1C](#)),⁶ and clone fraction distributions were followed over time ([Figure 1D](#)). We detected a significant reduction in the average clone fraction ([Figure 1E](#)) and observed a reduced number of fixed mutant clones ([Figure 1F](#)) in caffeine-treated mice. In line with our in vitro findings, wild-type ISC dynamics did not significantly change upon caffeine administration ([Supplementary Figure 2E–I](#)). Although the reduction in crypt fixation of *Apc*-mutant clones would suggest a subsequent reduction in adenoma development, long-term administration of caffeine does not impact the number and location of adenomas ([Figure 1G](#)). However, average adenoma size ([Figure 1H](#)) and the corresponding size distributions per animal ([Supplementary Figure 3A](#)

and *B*) suggest that adenomas in caffeine-treated mice are generally smaller, pointing toward a delay in adenoma development. Closer analysis of these adenomas reveals a significant increase in *Notum* and elevated *Wif1* expression in response to long-term caffeine treatment ([Figure 1I–L](#)), whereas *Dkk2* expression is unaltered ([Supplementary Figure 3C and D](#)). These findings suggest that long-term NOTUM inhibition results in a compensatory up-regulation of Wnt antagonists. In line with this observation, long-term (21 days) in vitro caffeine administration to *Apc*-mutant organoids resulted in elevated *Notum* expression and significant *Wif1* up-regulation ([Supplementary Figure 3E–G](#)). Moreover, CM transfer of *Apc*-mutant organoids pretreated with caffeine for 21 days failed to rescue wild-type clonogenicity compared with CM of short-term (4 days) treated *Apc*-mutant organoids ([Supplementary Figure 3H](#)). To strengthen these findings, we analyzed adenomas from *Notum*^{KO} mice ([Supplementary Figure 3I](#)).⁴ Previous work revealed that concomitant *Apc* and *Notum* loss reduces adenoma size and number but does not completely prevent adenoma formation, suggesting that *Notum* loss also delays tumor initiation.⁴ To assess whether this is caused by compensatory up-regulation of Wnt antagonists, we quantified the expression of the 3 most up-regulated Wnt antagonists detected in this model⁴: *Notum*, *Wif1*, and *Dkk3*. As expected, we observed a marked decrease in *Notum* expression in *Notum*^{KO} mice ([Supplementary Figure 3J and K](#)). Furthermore, *Notum*^{KO} adenomas display increased *Wif1* expression ([Supplementary Figure 3L and M](#)), whereas *Dkk3* expression remains unchanged ([Supplementary Figure 3N and O](#)). Together, our findings reveal that long-term NOTUM inhibition by caffeine or conditional *Notum* loss activates a feedback loop that facilitates increased expression of Wnt antagonists resulting in progression of adenoma formation ([Supplementary Figure 4](#)). We

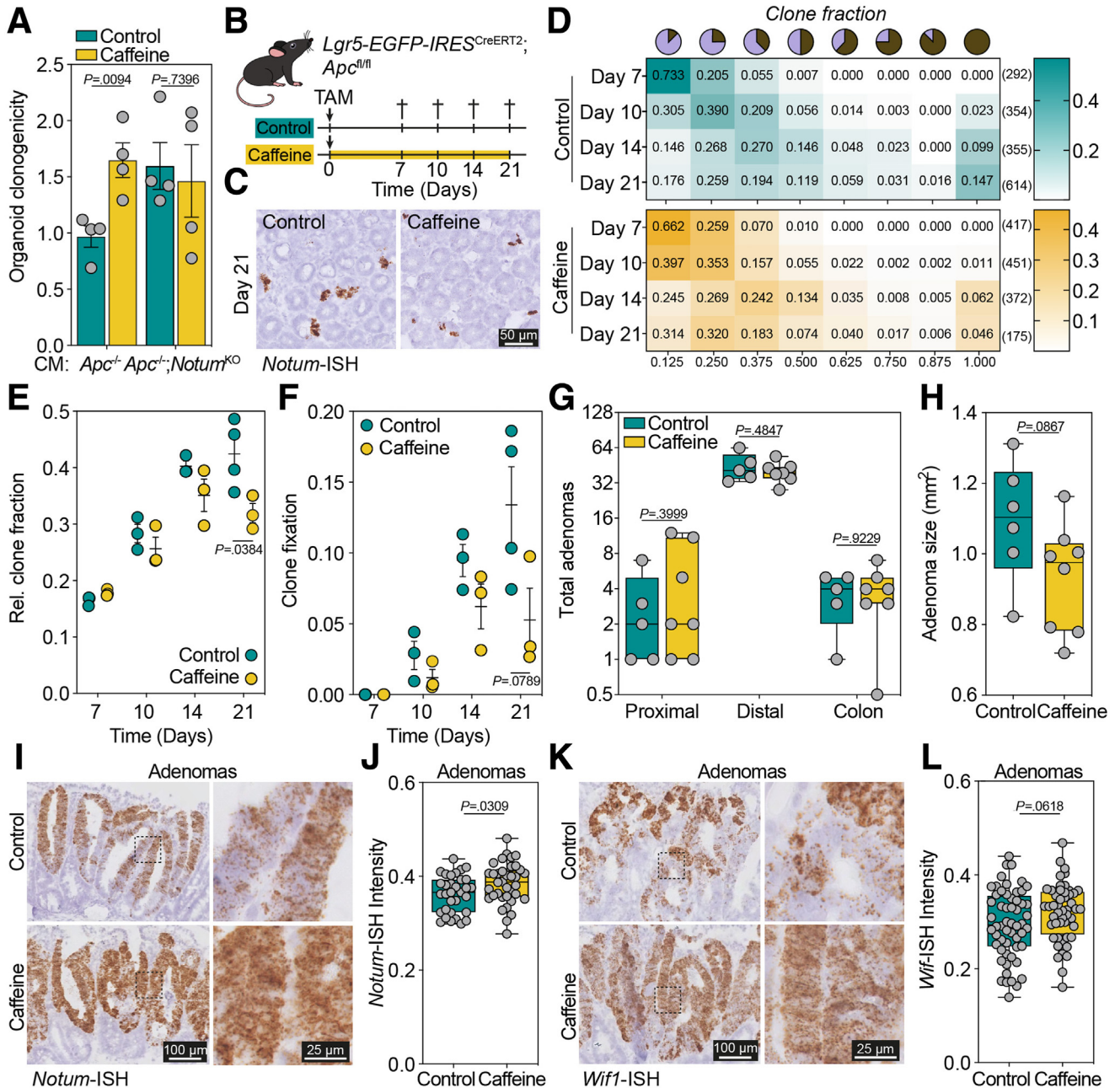


Figure 1. (A) Clonogenicity of WT organoids incubated with 40% *Apc*^{-/-} CM or *Apc*^{-/-}; *Notum*^{KO} CM with/without caffeine ($n = 4$). **(B)** Schematic illustration of in vivo experiment. **(C)** Detection of *Apc*-mutant clones using *Notum*-ISH. **(D–F)** Clone fraction distributions ($n =$ no. of crypts) **(D)**, average clone fraction **(E)**, and crypt fixation **(F)** of *Apc*-mutant clones in the absence/presence of caffeine ($n = 3–4$ mice per group). **(G and H)** Adenoma number per intestinal region **(G)**, average adenoma size in the distal SI **(H)** ($n = 5–7$ mice per group). **(I–L)** *Notum* **(I and J)** and *Wif1* **(K and L)** expression in adenomas of mice treated with/without caffeine ($n = 3$ mice, each dot is an ISH-positive region).

specifically observe up-regulation of *Notum* and *Wif1*, which work at the Wnt-ligand level, but not *Dkk2* and *Dkk3*, which interfere with co-receptors LRP5/6, suggesting that this feedback loop could function to control the bioavailability of Wnt ligands. Our findings are especially relevant

because of the vast global consumption of caffeinated beverages and the high prevalence of *APC* mutations in sporadic CRC. This study emphasizes the inherent difficulty of targeting the Wnt pathway as a cancer prevention strategy, and future research should consider the duration of caffeine

administration as well as dosing schedules to avoid compensation. Although it remains unclear whether caffeine is the only putative cancer protective ingredient in coffee,⁸ our results could potentially explain why coffee intake is associated with a reduced risk of CRC development⁹ and progression.¹⁰

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Conflicts of interest

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