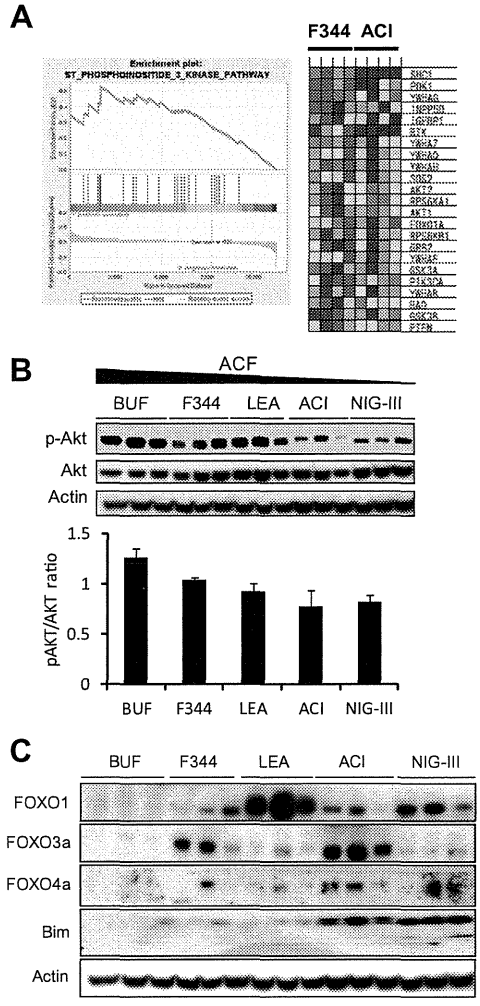

Fig. 3. Basal levels of pAKT in the colon correlate with predisposition to obesity and colon cancer. (A) Gene set enrichment analysis. The colonic crypts from F344 and ACI rats ($n = 4$ each) were subject to microarray analysis. Genes in the PI3K pathway overrepresented in differentially expressed genes (*left*). A heat map showing PI3K pathway genes (*right*). (B) Correlation between magnitude of AKT activation and the incidence of ACF. Western blotting analysis ($n = 3$) of the colonic crypts for total AKT, p-AKT (*upper panel*). Signal intensity ratio of p-AKT to total AKT correlated with the number of PhIP-induced ACF (*lower panel*). (C) Expression of pro-apoptotic molecules downstream of AKT. Western blotting analysis ($n = 3$) revealed FOXO family genes and Bim tended to show lower expression in tumor-prone rat strains. β-actin serves as a loading control.

observation, GSK-3βa key substrate of AKT, was phosphorylated specifically in the same group. The increase of pAKT was marginal without PhIP treatment or under HFD, even after PhIP treatment. To determine if the activation is achieved by a direct effect of PhIP on colonic cells, we treated human normal colon cells FHC with 10 μM of acetoxy-PhIP, a biologically active form of PhIP. Phosphorylation of AKT was indeed observed *in vitro*, albeit at a very early point and in a transient manner (Fig. 4B). These results implied that PhIP directly and promptly activates AKT, which could be sustained *in vivo* only under LFD, by an unknown mechanism.

3.5. PhIP and HFD inhibited apoptosis and activated Wnt pathway by distinct mechanisms

Given that GSK-3β promotes degradation of β-catenin, inactivation of GSK-3β by AKT is supposed to result in β-catenin accumulation leading to Wnt pathway activation. Indeed, PhIP-induced AKT activation increased the amount of total β-catenin, consistent with an earlier study [19], but to a lesser extent compared to HFD

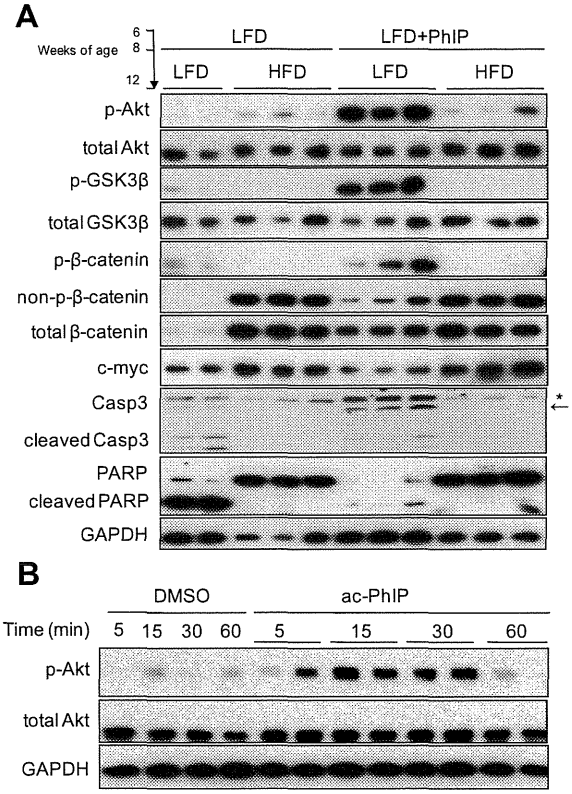

Fig. 4. Wnt pathway activation and inhibition of apoptosis by PhIP and HFD. (A) Characterization of key molecules in the Wnt pathway and apoptosis. Colonic crypts of the BUF rats from 4 subgroups ($n = 3$ each, $n = 2$ for LFD with PhIP) were analyzed by Western blotting analysis. GAPDH serves as a loading control. Non-specific bands (*asterisk*), specific bands (*arrow*) for full-length caspase3. Note that effects by PhIP were sustained even at 4 weeks later under LFD, but not under HFD. (B) Activation of AKT by PhIP in vitro. Normal human colon cells FHC were exposed to 10 μM N-acetoxy-PhIP (ac-PhIP). Negative controls were treated with DMSO ($n = 1$). Cells were collected at 5, 15, 30, and 60 min after PhIP treatment ($n = 2$).

(Fig. 4A). To qualitatively characterize β-catenin, we examined its phosphorylated and non-phosphorylated form, corresponding to an inactive and active form, respectively. It was revealed that HFD exclusively increased the amount of active β-catenin, while PhIP predominantly increased the amount of inactive β-catenin. In line with this observation, the level of c-myc, a major Wnt target gene, was indeed higher under HFD than upon PhIP treatment (Fig. 4A). AKT-induced elevation of inactive β-catenin, however, contradicts with the assumption that inactivation of GSK-3β should result in accumulation of active β-catenin, strongly suggesting that an alternative mechanism might be operating.

We next examined the effects of PhIP and HFD on apoptosis. Caspase3 and poly-ADP ribose polymerase (PARP) were predominantly in cleaved forms in colonic crypts under LFD, indicating massive apoptosis. By contrast, the cleaved forms were not detected under HFD or treated by PhIP, which seems to be achieved via distinct mechanisms. HFD suppressed expression of caspase3, thereby diminishing its cleaved form, while PhIP suppressed cleavage from full-length caspase3. Conversely, PhIP regulated PARP and caspase3 in a reciprocal manner (Fig. 4A). Collectively, HFD and PhIP activated the Wnt pathway and inhibited apoptosis, but through distinct mechanisms in the colon.

4. Discussion

Obesity, a major risk for CRC, has been generally implicated in progression from the initiation step of carcinogenesis. In the

present study, we showed that obesity could be also implicated in the early stages, by sharing a common genetic predisposition with PhIP-induced tumorigenesis. The common genetic predisposition appeared to be conveniently estimated by the level of serum TG and activated AKT in the colonic mucosa. AKT was also dynamically activated by PhIP, which seemed to be promoted by the intestinal microenvironment, but inhibited by HFD, underscoring the relevance of cooperation between genetic and environmental factors toward PhIP-induced colon carcinogenesis. Given the pro-survival properties of AKT and pro-tumorigenic effects of obesity, inhibition of AKT activation by HFD appears paradoxical in terms of tumor promotion. However, this observation might account for the reason why a cycling protocol alternating PhIP with HFD could induce colon tumors more efficiently than continuous exposure to PhIP [11].

PhIP-induced activation of AKT resulted in inactivation of GSK-3β as predicted, but did not lead to full activation of β-catenin for an unknown reason. Consequently, PhIP + LFD induced only a modest increase of the non-phosphorylated β-catenin compared to HFD. These results imply Wnt pathway-independent roles of GSK-3β inhibition in PhIP-induced tumorigenesis. In support of this notion, the colony formation potential of singly dissociated intestinal stem cells in 3D culture is significantly improved by a GSK-3β inhibitor, but not by Wnt3a ligands [20], raising the possibility that PhIP could promote survival of stem cells that might harbor mutations introduced by PhIP. Both PhIP and HFD inhibited apoptosis of colonic crypts, but surprisingly in a completely distinct manner that has never been reported previously. Expression of PARP was suppressed by PhIP, but induced and retained by HFD. As PARP is a component of the TCF4/β-catenin complex and positively regulates its transcriptional activity [21,22], its presence might contribute to a more pronounced activation of the Wnt pathway by HFD than by PhIP.

Considering high serum TG has recently emerged as a high risk factor for CRC in humans [23] [24], consistent with the present study, the findings from this study might have implications on personalized medicine. For instance, those individuals with high serum TG and AKT phosphorylation in the colon might constitute a subgroup with higher risk for CRC, even in the absence of macroscopic colonic lesions. Development of biomarkers for downstream of AKT would be also warranted, which would enable efficient reduction of cancer risk by patient education, early detection of cancer and therapeutic intervention. Taken together, we demonstrated the relevance of AKT in the development of PhIP-induced and obesity-related CRC, providing not only mechanistic insights, but also clinical implications on the diagnosis and prevention of CRC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.059.

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