RNA-binding protein HuR promotes growth of small intestinal mucosa by activating the Wnt signaling pathway

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ABSTRACT Inhibition of growth of the intestinal epithelium, a rapidly self-renewing tissue, is commonly found in various critical disorders. The RNA-binding protein HuR is highly expressed in the gut mucosa and modulates the stability and translation of target mRNAs, but its exact biological function in the intestinal epithelium remains unclear. Here, we investigated the role of HuR in intestinal homeostasis using a genetic model and further defined its target mRNAs. Targeted deletion of HuR in intestinal epithelial cells caused significant mucosal atrophy in the small intestine, as indicated by decreased cell proliferation within the crypts and subsequent shrinkages of crypts and villi. In addition, the HuR-deficient intestinal epithelium also displayed decreased regenerative potential of crypt progenitors after exposure to irradiation. HuR deficiency decreased expression of the Wnt coreceptor LDL receptor-related protein 6 (LRP6) in the mucosal tissues. At the molecular level, HuR was found to bind the Lrp6 mRNA via its 3′-untranslated region and enhanced LRP6 expression by stabilizing Lrp6 mRNA and stimulating its translation. These results indicate that HuR is essential for normal mucosal growth in the small intestine by altering Wnt signals through up-regulation of LRP6 expression and stimulating a novel role of HuR deficiency in the pathogenesis of intestinal mucosal atrophy under pathological conditions.

INTRODUCTION

The mammalian intestinal epithelium is among the most rapidly self-renewing tissues in the body, and its integrity is preserved through strict regulation of cell proliferation, migration, differentiation, and apoptosis (Wang, 2007; Sato and Clevers, 2013; Xiao et al., 2013).

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-03-0853) on August 27, 2014.
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Abbreviations used: Fzd7, Frizzled-7; IEC, intestinal epithelial cell; IE-HuR−/−, intestinal epithelial-specific HuR deletion; IP, immunoprecipitation; LRP6, LDL receptor–related protein 6; RBP, RNA-binding protein; RNP, ribonucleoprotein; UTR, untranslated region; Wnt3a-IECs, Wnt3a-transfected IEC-6 cells.
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Undifferentiated intestinal epithelial cells (IECs) replicate rapidly in the proliferative zone within crypts and differentiate as they migrate up the luminal surface to replace lost cells (Sato and Clevers, 2013). Apoptosis occurs in both the crypt area, where this process maintains the balance in cell number between newly divided and surviving cells, and the luminal surface of the intestine, where differentiated cells are lost (Gunther et al., 2013). The epithelium of the human small intestine undergoes ~1011 mitoses/d, and this dynamic turnover rate is tightly regulated by numerous factors at multiple levels in order to maintain proper homeostasis (Johnson, 1988; Wang, 2007; Gunther et al., 2013). Inhibition of intestinal mucosal growth occurs commonly in various critical disorders, particularly in patients who undergo massive surgical operations and are then supported with total parenteral nutrition (Wildhaber et al., 2003; Puleo et al., 2011). However, the exact mechanisms underlying the control of intestinal epithelial integrity, especially at the posttranscriptional level, remain largely unknown.

Supplemental Material can be found at: http://www.molbiolcell.org/content/suppl/2014/08/25/mbc.E14-03-0853v1.DC1

Molecular Biology of the Cell
The regulation of mRNA stability and translation is a major mechanism by which mammalian cells control gene expression (Garneau et al., 2007). The posttranscriptional fate of a given mRNA is predominantly governed by the interaction of specific mRNA sequences (cis-elements) with two major types of trans-acting factors: RNA-binding proteins (RBPs) and microRNAs (miRNAs); Keene, 2007; Zhang et al., 2009; Mendell et al., 2012). Ribonucleoprotein (RNP) associations regulate the intracellular transport of the mRNA and its association with the translation and decay machineries (Keene, 2007; Houseley and Tollervey, 2009; Zou et al., 2010). Many labile mRNAs contain relatively long 3′-untranslated regions (3′-UTRs) bearing sequences (e.g., U- and AU-rich elements [AREs]) that function as determinants of mRNA stability and translation (Gherzi et al., 2004; Keene, 2007). Among the RBPs that regulate specific subsets of mRNAs are several RBPs that modulate mRNA turnover (HuR, NF90, AUF1, BRF1, TTP, KSRP) and RBPs that modulate translation (HuR, TIAR, NF90, TIA-1; Gherzi et al., 2004; Keene, 2007; Lee et al., 2010). In cells responding to proliferative, immune, and stress-causing stimuli, RBPs bind to the specific sequences in the 3′-UTRs or coding regions (CRs) of collections of target mRNAs and alter their turnover and translation rates (Liao et al., 2007; Lee et al., 2010; Yu et al., 2013). In addition, RBPs and miRNAs can jointly regulate shared target mRNAs (Srikantan et al., 2012). For example, HuR competes with miR-195 to modulate Stmn1 mRNA stability antagonistically (Zhuang et al., 2013), whereas CUG-binding protein 1 (CUGBP1) and miR-222 repress Cdk4 mRNA translation synergistically (Xiao et al., 2011).

The ubiquitous RBP HuR plays a role in the posttranscriptional control of mRNAs bearing AREs (Abdelmohsen et al., 2007; Papadaki et al., 2009). Because constitutive HuR inactivation in vivo is lethal to embryos (Katsanou et al., 2009), most of our knowledge about HuR function comes from studies conducted in cultured cells and/or experimental conditions in which the levels of HuR are artificially increased through the use of transgenic mice. In this regard, overexpression of HuR in mouse macrophages is shown to modulate the translation of selective inflammatory mRNAs (Katsanou et al., 2005), and fertility is compromised in mice overexpressing HuR in testis (Levadoux-Martin et al., 2003). Several studies using conditional HuR-knockout mice further revealed that myeloid deletion of HuR exacerbates the production of proinflammatory cytokines and increases the sensitivity to acute inflammatory reactions such as endotoxemia (Yiakouvaki et al., 2010). In cells responding to proliferative, immune, and stress-causing stimuli, RBPs bind to the specific sequences in the 3′-UTRs or coding regions (CRs) of collections of target mRNAs and alter their turnover and translation rates (Liao et al., 2007; Lee et al., 2010; Yu et al., 2013). In addition, RBPs and miRNAs can jointly regulate shared target mRNAs (Srikantan et al., 2012). For example, HuR competes with miR-195 to modulate Stmn1 mRNA stability antagonistically (Zhuang et al., 2013), whereas CUG-binding protein 1 (CUGBP1) and miR-222 repress Cdk4 mRNA translation synergistically (Xiao et al., 2011).

RESULTS

Tissue-specific HuR deletion results in small intestinal mucosal atrophy

To investigate the in vivo function of HuR in intestinal epithelium, we generated intestinal epithelium-specific HuR deletion (IE-HuR<sup>+/−</sup>) mice by crossing HuR<sup>fl/fl</sup> mice with carrying Villin-Cre (Supplemental Figure S1A). As described previously (Mosmann et al., 1983; Katsanou et al., 2009), HuR<sup>fl/fl</sup> mice were produced via standard gene-targeting procedures in embryonic stem cells and contained a fully functional HuR allele. Heterozygous IE-HuR<sup>+/−</sup> mice appeared phenotypically normal and were subsequently intercrossed for the generation of homozygous IE-HuR<sup>−/−</sup> mice. Age-matched IE-HuR<sup>−/−</sup> mice and littermate control mice (3 or 4 mo old) were used for comparison of phenotype and showed HuR deletion at genomic DNA isolated from the intestinal mucosa of IE-HuR<sup>−/−</sup> animals (Figure 1A).

**FIGURE 1.** Characterization of intestinal epithelium-specific HuR deletion (IE-HuR<sup>−/−</sup>) mice. (A) PCR analysis of genomic DNA from small intestinal mucosa indicates floxed, HuR deletion, and Cre bands in genomic DNA isolated from the intestinal mucosa of IE-HuR<sup>−/−</sup> animals (Figure 1A).
Consistent with this finding, HuR mRNA and protein in the small intestinal and colonic mucosa were undetectable in IE-HuR\(^{-/-}\) mice (Figure 1B), whereas HuR expression levels in the intestinal mucosa of HuR\(^{floxed/Cre}\) and HuR\(^{+/+}\) mice were normal. Immunohistochemical staining assays revealed that HuR levels almost completely disappeared in epithelial cells in the intestinal mucosa of IE-HuR\(^{-/-}\) mouse, but its expression was unaffected in submucosal connective tissue (Figure 1C). On the other hand, there were no changes in HuR expression levels in stomach mucosa, lung, liver, and pancreas in IE-HuR\(^{-/-}\) mice compared with those observed in littermates (Supplemental Figure S1, B and C). These findings suggest that the IE-HuR\(^{-/-}\) mouse is a suitable gene-targeting model of HuR deficiency in the intestinal epithelium.

Generally, IE-HuR\(^{-/-}\) mice looked normal; there were no significant differences in body weight (Figure 2A), gastrointestinal gross morphology (Figure 2B), reproduction, and general appearances between IE-HuR\(^{-/-}\) mice and littermate controls. Of interest, IE-HuR\(^{-/-}\) mice exhibited significant mucosal atrophy in the small intestine, as indicated by a decrease in the lengths of villi and crypts (Figure 2, C and D). The proliferating crypt cell population, marked by bromodeoxyuridine (BrdU; S phase), decreased remarkably in the small intestine of HuR\(^{-/-}\) mice compared with those from littermates (Figure 2E). Accordingly, the levels of cell proliferation marker proteins proliferating cell nuclear antigen (PCNA) and Ki67 were also decreased in the small intestinal mucosa of IE-HuR\(^{-/-}\) mice (Figure 2F). Moreover, the loss of HuR in IECs inhibited the regenerative potential of crypt progenitors, since S-phase descendants in the villous regions decreased significantly in IE-HuR\(^{-/-}\) mice compared with those observed in control littermates after exposure to irradiation (Figure 3, A and B). Consistently, the villus/crypt ratio in IE-HuR\(^{-/-}\) mice also decreased when measured 10 h after irradiation (Figure 3C). We also examined changes in colonic mucosal growth in IE-HuR\(^{-/-}\) mice and found that epithelium-specific HuR deletion did not alter mucosal growth in the colon. There were no significant decreases in the lengths of villi and crypts and BrdU-labeled cell proliferation in IE-HuR\(^{-/-}\) mice compared with littermate controls (unpublished data). In addition, specific HuR deletion in IECs did not affect lineage differentiation in the intestine (Supplemental Figure S2, A and B), gut permeability (Supplemental Figure S2C), or crypt number per tissue area (Supplemental Figure S2D).
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These results indicate that conditional HuR deletion in IECs results in small intestinal mucosal atrophy but does not alter colonic mucosal growth.

HuR interacts with the 3′-untranslated region of Lrp6 mRNA

To investigate the mediators of the HuR-elicted effects, we found that the HuR-deficient intestinal epithelium was associated with decreased levels of Lrp6 mRNA and LDL-receptor-related protein 6 (LRP6; Figure 4, A–C). In contrast, HuR deletion increased expression of p65 and Smad7 in the intestinal mucosa, although it failed to alter the levels of Frizzled-7 (Fzd7) or E-cadherin (Figure 4D). Because there are several potential hits of HuR motif in the Lrp6 mRNA, we further examined whether HuR directly interacted with the Lrp6 mRNA in cultured IEC-6 cells by performing ribonucleoprotein immunoprecipitation (RIP) assays using anti-HuR antibody under conditions that preserved RNP integrity (Lal et al., 2004). The interaction of Lrp6 mRNA with HuR was examined by isolating RNA from the immunoprecipitated material and subjecting it to reverse transcription (RT), followed by either conventional PCR or real-time quantitative PCR (qPCR) analyses. As shown in Figure 5A, the Lrp6 PCR products were highly enriched in HuR samples compared with control immunoglobulin G (IgG) samples. HuR was also found to bind the p65 mRNA, although it did not preferentially associate with Fzd7, E-cad, and Smad7 mRNAs (Supplemental Figure S3). To determine whether HuR binds to specific regions of the Lrp6 5′-UTR, CR, and 3′-UTR, we further tested [HuR/Lrp6 mRNA] associations by using biotinylated transcripts that spanned the Lrp6 mRNA regions shown (Figure 5B, schematic). After incubation with cytoplasmic lysates, the interaction between the biotinylated Lrp6 transcripts and HuR was examined by biotin pull-down, followed by Western blot analysis (Abdelmohsen et al., 2007; Chen et al., 2008). As shown, HuR readily associated with the Lrp6 3′-UTR, particularly the fragment 3′UTR-F2 (spanning positions 5881–6441), but not with Lrp6 5′-UTR and fragments of CR transcripts. Moreover, the abundance of [HuR/Lrp6 mRNA] complexes was enriched in the intestinal mucosa isolated from littermate control mice but not in the mucosa from IE-HuR−/− mice, as measured by RIP/qPCR analysis (Figure 5C). These results indicate that the Lrp6 mRNA is a novel target of HuR in the intestinal epithelium.

HuR regulates LRP6 expression posttranscriptionally

To determine the functional consequences of [HuR/Lrp6 mRNA] associations, we reduced HuR levels by small interfering RNA (siRNA) targeting the HuR mRNA (siHuR), as reported previously (Liu et al., 2009). IEC-6 cells transfected with siHuR showed <10% of HuR levels as compared with the levels seen in cells transfected with control siRNA (C-siRNA; Figure 6A, top). Of importance, HuR silencing reduced LRP6 protein by ~85% (Figure 6A, middle), but it decreased Lrp6 mRNA levels by only ~40% (Figure 6B). The reduction in Lrp6 mRNA by HuR silencing was due to the destabilization of Lrp6 mRNA, since silencing HuR selectively lowered the Lrp6 mRNA half-life (Figure 6C). To examine whether HuR silencing also alters the translation of Lrp6 mRNA, we examined changes in the level of new LRP6 protein synthesis after transfection with siHuR and demonstrated that newly synthesized LRP6 protein decreased significantly in HuR-silent cells compared with cells transfected with C-siRNA (Figure 6D). Inhibition of LRP6 protein synthesis by HuR silencing was specific, since there was no change in nascent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) synthesis after transfection with siHuR. To further define the role of HuR in the regulation of LRP6 translation, we examined the relative distribution of Lrp6 mRNA in individual fractions from polyribosome gradients. Although decreasing the levels of HuR did not affect global polysomal profiles (unpublished data), the abundance of Lrp6 mRNA associated with actively translating fractions (fractions 9–11) decreased in HuR-silent cells with a shift of Lrp6 mRNAs to low-translating fractions (fractions 6–8; Figure 6E, top); it is important to note that even small shifts in polysome size can reflect large changes in protein synthesis. In contrast, Gapdh mRNA, encoding the housekeeping protein GAPDH, distributed similarly in both groups (Figure 6E, bottom). HuR regulates the stability and translation of Lrp6 mRNA by interacting with Lrp6 3′-UTR, since HuR silencing decreased the levels of Lrp6-3′UTR-F2 luciferase reporter activity (Figure 6F) but did not affect the activities of Luc-5′-UTR and Luc-CR-F1 reporter genes. On the other hand, ectopic
overexpression of HuR by infection with the adenoviral vector containing the corresponding HuR cDNA (AdHuR) increased LRP6 expression by increasing Lrp6 mRNA stability and translation via interaction with its 3′-UTR (Supplemental Figure S4). These results indicate that HuR increases LRP6 expression by stabilizing the Lrp6 mRNA and enhancing its translation.

HuR silencing inhibits IEC proliferation in vitro

To investigate the consequences of HuR-regulated LRP6 expression upon cell proliferation, we used stable Wnt3a-transfected IEC-6 cells (Wnt3a-IECs) that were recently developed in our laboratory (Liu et al., 2012). Stably transfected Wnt3a-IECs expressed high levels of Wnt3a protein (Figure 7Aa) and exhibited a dramatic activation of Wnt/β-catenin signaling pathway, as indicated by an increase in the levels of the Wnt/β-catenin reporter plasmid superTOPFLASH activity (Figure 7Ab). Consistently, ectopic Wnt3a overexpression increased IEC proliferation (Figure 7Ac), although the levels of HuR and LRP6 were unchanged in Wnt3a-IECs (Figure 7B, left). Cell cycle analysis indicated that ~35% of parent IECs were in the S phase, whereas the population of S-phase cells significantly increased to ~54% in stable Wnt3a-IECs, along with a decrease in G1-phase cells. Moreover, decreased levels of endogenous LRP6 in stable Wnt3a-IECs by HuR silencing decreased Wnt/β-catenin signaling activity (Figure 7Ca), inhibited cell growth (Figure 7Cb), and returned the cell cycle distribution displayed by parent IECs. When stable Wnt3a-IECs were transfected with siHuR, S-phase cells decreased to ~27% from ~53% in cells transfected with C-siRNA (Figure 7C, D and E). The inhibitory effects of decreasing endogenous LRP6 in stable Wnt3a-IECs by HuR silencing were not simply due to clonal variation, since two different clonal populations, Wnt3a-IEC-C1 and Wnt3a-IEC-C2, showed similar responses. LRP6 silencing in stable Wnt3a-IECs by transfection with siLRP6 also resulted in inhibition of the Wnt/β-catenin signaling pathway and cell growth, as indicated by decrease in S-phase cells. Conversely, ectopic overexpression of LRP6 in HuR-silenced cells by transfection with the LRP6 expression vector increased cell growth, along with increase in S-phase cells (Supplemental Figure S5). Marginal inhibition of parental IEC-6 cell proliferation by silencing HuR or LRP6 was also observed (Supplemental Figure S6). On the other hand, silencing HuR or LRP6 alone failed to induce apoptosis in parent IEC-6 cells or Wnt3a-IECs, as measured by assessment of cell viability, annexin-V staining, and levels of cleaved caspase-3. These results indicate that HuR-induced LRP6 expression plays an important role in stimulation of IEC proliferation.

**DISCUSSION**

Our previous studies demonstrated that HuR in IECs regulates expression of several proliferation/apoptosis-associated genes, such as p53 and nucleophosmin (Zou et al., 2006), ATF2 (Xiao et al., 2007), XIAP (Zhang et al., 2009), JunD (Zou et al., 2009), c-Myc (Liu et al., 2010), MEK-1 (Wang et al., 2010), c-Myc (Liu et al., 2009), and Stim1 (Zhuang et al., 2013), at the posttranscriptional level, suggesting the involvement of HuR in maintaining intestinal epithelial homeostasis. However, all of these studies were conducted in cultured IECs; therefore the exact function of HuR in the intestinal epithelium in vivo remains to be fully defined. Using a conditional tissue-specific gene-targeting approach, here we provided powerful genetic evidence of the physiological role of HuR in the regulation of intestinal mucosal integrity. Specific deletion of HuR in IECs caused small intestinal mucosal atrophy in mice, although it failed to alter colonic mucosal growth. Experiments aimed at characterizing the aspects of HuR targets in this process suggested that the inhibition of intestinal mucosal growth induced by HuR deletion resulted from the inactivation of Wnt signaling due to the repression of the expression of the Wnt co-receptor LRP6. These findings advance our understanding of the biological function of HuR in the intestinal epithelium and highlight a novel role of HuR deficiency in...
HuR promotes growth of intestinal mucosa

Results from our in vivo studies demonstrate the essential role of HuR in normal mucosal growth in the small intestine, since significant defects in mucosal renewal occurred in IE-HuR−/− mice, as indicated by a decrease in cell proliferation in the crypts and subsequent shrinkages of crypts and villi in the mucosal tissue. This inhibitory phenotype in small intestinal mucosal growth by specific HuR deletion is not surprising, because HuR up-regulates expression of the pathogenesis of intestinal mucosal atrophy under pathological conditions.

Several recent mouse HuR gene-deletion studies in diverse tissue/organ cell types have improved our understanding of physiological roles of HuR in mammals that, in some circumstances, contradicts conventional wisdom of previous in vitro cell biology studies (Xiao et al., 2007; Katasanou et al., 2009; Papadaki et al., 2009; Young et al., 2009). Results from our in vivo studies demonstrate the essential role of HuR in normal mucosal growth in the small intestine, since significant defects in mucosal renewal occurred in IE-HuR−/− mice, as indicated by a decrease in cell proliferation in the crypts and subsequent shrinkages of crypts and villi in the mucosal tissue. This inhibitory phenotype in small intestinal mucosal growth by specific HuR deletion is not surprising, because HuR up-regulates expression of the pathogenesis of intestinal mucosal atrophy under pathological conditions.

FIGURE 5: HuR binds the 3′-UTR of Lrp6 mRNA. (A) Association of endogenous HuR with endogenous Lrp6 mRNA in IEC-6 cells as measured by RIP/qPCR analysis using either anti-HuR antibody (Ab) or control IgG: (a) Lrp6 mRNAs in HuR IP as measured by RT-PCR (left) and qPCR (right) analyses; and (b) levels of total input mRNAs. Values are the means ± SEM from triplicate samples. *p < 0.05 compared with IgG IP. (B) HuR immunoblots using the pull-down materials by biotinylated transcripts of Lrp6 5′-UTR, CR, and 3′-UTR. Left, schematic representation of various biotinylated Lrp6 transcripts. (C) Association of HuR with the Lrp6 mRNA in small intestinal mucosa in littermates and IE-HuR−/− mice as measured by RIP/qPCR analysis. Values are the means ± SEM (n = 5). *p < 0.05 compared with littermates.
c-Myc, which is crucial for stimulation of IEC proliferation and enhances gut mucosal healing after injury (Wang and Johnson, 1994; Liu et al., 2005). Consistent with our findings, a recent study (Giammanco et al., 2014) shows that conditional tissue-specific genetic deletion of HuR in IECs reduces intestinal tumor development.
HuR can act as a proapoptotic or antiapoptotic factor, depending on cell type, magnitude of damage, type of apoptotic inducers, and the presence or absence of other factors, the effect of HuR deletion on IEC apoptosis in vivo will be investigated separately.

by altering distinct signaling pathways of proliferation and apoptosis. Although the present study is tightly focused on the role of HuR in regulating intestinal mucosal growth, HuR-modulated apoptosis in IECs is also implicated in intestinal epithelial homeostasis. Because HuR can act as a proapoptotic or antiapoptotic factor, depending on cell type, magnitude of damage, type of apoptotic inducers, and the presence or absence of other factors, the effect of HuR deletion on IEC apoptosis in vivo will be investigated separately.
Although HuR levels were undetectable in the colonic epithelium of IE-HuR−/− mice, there were no significant differences in the rates of colonic mucosal growth between HuR-deficient mice and littermates. The exact reasons for which HuR depletion failed to alter colonic mucosal growth remain unknown, but basal mucosal turnover rate in the colon is lower than that observed in the small intestine (Johnson, 1988). In addition, the basal level of LRP6 (a major target of HuR in the small intestinal epithelium) was markedly lower in the colonic mucosa than in the small intestinal mucosa (unpublished data). Because limited information is available on the specific mechanisms underlying the control of mucosal growth throughout different sections of the intestinal tract, the relative importance of Wnt signaling, particularly possible alternatives for LRP6 in colonic mucosa, are unclear. Adenomatous polyposis coli (APC) protein was recently shown to function as an RNA-binding protein, and the collection of interacting transcripts provides a link between Wnt pathway proteins and RNA networks (Preitner et al., 2014). However, the exact role of APC in colonic mucosal homeostasis in HuR-deficient mice remains to be investigated.

Another significant finding from this study is that the Lrp6 mRNA is a novel target of HuR and that [HuR/Lrp6 mRNA] association not only stabilizes Lrp6 mRNA but also enhances its translation. Through the use of various ectopic reporters bearing partial transcripts spanning the Lrp6 5′-UTR, CR, and 3′-UTR with or without the predicted HuR-hit motif, our results further show that HuR interacted predominantly with the Lrp6 3′-UTR F2 element (spanning positions 5881–6441) but not with the 5′-UTR or CR elements. Although we did not characterize the specific nucleotides with which HuR interacts and increases Lrp6 mRNA stability and translation, there are four predicted HuR-hit motifs within the Lrp6 3′-UTR F2 region. Moreover, the F2 sequence of the Lrp6 3′-UTR was found to be functional, because both repression of LRP6 by HuR silencing and stimulation of reporter activity by HuR overexpression occurred only when cells were transfected with the Lrp6-3′-UTR-F2 luciferase reporter constructs but not with the Lrp6-5′-UTR or Lrp6 CR-F1 reporter constructs. These observations are consistent with other results, which demonstrated that HuR associates with its target mRNAs via their 3′-UTRs, thus stabilizing mRNAs and/or increasing their translation (La et al., 2004; Xiao et al., 2007; Wang et al., 2010). Although HuR commonly interacts with the 3′-UTRs of target transcripts, in some instances it also associates with the CRs of target mRNAs for its regulatory actions. In this regard, we reported that HuR stabilizes Xiap mRNA by directly interacting with the Xiap CR (Zhang et al., 2009).

Intestinal epithelial–specific HuR deletion inhibits small intestinal mucosal growth by inactivating Wnt signaling as a result of repression of LRP6 expression. Wnt signaling is critically required for gut development and acts as a key regulator of intestinal epithelial renewal (van Es et al., 2005; Liu et al., 2012). Central to this signaling pathway is the stabilization of β-catenin and its interaction with DNA-binding factors of the T-cell factor (TCF) family in the nucleus (Clevers, 2006). In response to stress, released Wnt proteins in the extracellular milieu bind to serpentine receptors of the Fzd family and to coreceptors LRPS/6, which leads to accumulation of dephosphorylated β-catenin and its stabilization (He et al., 2004; Clevers, 2006). Subsequently, the stabilized β-catenin undergoes nuclear translocation and association with TCF transcription factors, enabling transactivation of their target genes. Conditional Wnt gene deletion or overexpression of the Wnt natural inhibitor Dickkopf1 disrupts gut development, represses mucosal growth, and delays healing of damaged mucosa (van Es et al., 2005; Koch et al., 2009). Activation of Wnt signaling also modulates the sensitivity of cells to apoptosis by altering c-Myc expression (Yoo et al., 2002). In this study, LRP6 expression levels decreased dramatically in the intestinal mucosal tissue in IE-HuR−/− mice and in an HuR-silent population of IEC-6 cells. Moreover, LRP6 silencing or decreased levels of LRP6 by HuR silencing inhibited Wnt/β-catenin signaling pathway and repressed cell proliferation in stable Wnt3a-IECs, whereas ectopic overexpression of LRP6 in HuR-silenced cells promoted cell proliferation. In sum, our results indicate that HuR is essential for the maintenance of intestinal mucosal homeostasis by altering Wnt signaling activity through posttranscriptional regulation of LRP6 expression.

MATERIALS AND METHODS

Animal studies

All experiments were approved according to animal experimental ethics committee guidelines by the University of Maryland Baltimore Institutional Animal Care and Use Committee. Mice were housed and handled in a specific pathogen-free breeding barrier and cared for by trained technicians and veterinarians. The strategy to generate and genotype IE-HuR−/− mice is provided in Supplemental Figure S1A. The HuR floxed mouse has been described elsewhere (Katasanou et al., 2009; Chi et al., 2011; Yiakoukaki et al., 2012), and HuRlox/lox (HuRflk) was crossed with Villin-Cre mice (kindly provided by Deborah Gumucio, University of Michigan, Ann Arbor, MI) to yield IE-HuR−/− mice. HuRflk-Cre− mice served as littermate control. Animals were killed by pentobarbital overdose. BrdU was incorporated in intestinal mucosa by intraperitoneal injection of 2 mg of BrdU (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline. A 4-cm small intestinal segment taken from 0.5 cm distal to the ligation of Treitz and the segment of middle colon were collected 1 h after injection. The mucosa was scraped from the underlying smooth muscle with a glass microscope slide and used for measurements of the levels of various mRNA and protein expression and HuR association with given mRNAs.

Cell cultures and plasmid construction

The IEC-6 cell line, derived from normal rat intestinal crypt cells, was used at passages 15–20 in experiments (Liu et al., 2003). Antibodies recognizing HuR, LRP6, Frizzled-7 (Fzd7), Smad7, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences (Sparks, MD), and the secondary antibody was obtained from Sigma-Aldrich. Stable Wnt3a-transfected IEC-6 cells (Wnt3a-IECs) were developed and maintained as described in our previous studies (Liu et al., 2012).

Recombinant adenoviral plasmids containing human HuR (Ad-HuR) were constructed by using the Adeno-X Expression System (Clontech, Mountain View, CA; Zou et al., 2006). The chimeric firefly luciferase reporter construct containing Lrp6 mRNA was described previously (Liu et al., 2009). The full-length Lrp6 5′-UTR, two fragments of CR, and different 3′-UTR fragments were subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) to generate the pmirGLO-Luc-Lrp6-5′-UTR, pmirGLO-Luc-Lrp6-3′-CR, and pmirGLO-Lrp6-3′-UTR reporter constructs. The Wnt/β-catenin signaling luciferase reporter plasmid superTOPFLASH was purchased from Addgene (Cambridge, MA). Transient transfections were performed using Lipofectamine reagent as recommended by the manufacturer, and the levels of firefly luciferase activity were normalized to Renilla luciferase activity. All of the primer sequences for generating these constructs are provided in Supplemental Table S1.

Western blot analysis

Whole-cell lysates were prepared using 2% SDS, sonicated, and centrifuged at 4°C for 15 min. The supernatants were boiled
and size-fractionated by SDS–PAGE. After the blots were incubated with primary antibody and then secondary antibodies, immunocomplexes were developed by using chemiluminescence.

RT followed by PCR and real-time qPCR analyses
Total RNA was isolated from cells after different treatments by using RNeasy Mini Kit (Qiagen, Valencia, CA) and used in reverse transcription and PCR amplification reactions as described (Xiao et al., 2011). The levels of Gapdh PCR product were assessed to monitor the even RNA input in RT or qPCR samples. qPCR was performed using 7500-Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA) with specific primers, probes, and software (Applied Biosystems).

Biotin pull-down assays and RIP analysis
The synthesis of biotinylated transcripts and analysis of HuR bound to biotinylated RNA were carried out as previously described (Xiao et al., 2007; Yu et al., 2011). cDNA from IEC-6 cells was used as a template for PCR amplification of 5′-UTR, CR, and 3′-UTR of Lrp6 mRNA. The 5′ primers contained the T7 RNA polymerase promoter sequence (T7; CCAAGCT-TCTAATACGAC-TCACATATGGAGA). All sequences of oligonucleotides for preparation of full-length Lrp6 5′-UTR and various fragments of CR or 3′-UTR are described in Supplemental Table S1. PCR-amplified products were used as templates to transcribe biotinylated RNAs by using T7 RNA polymerase in the presence of biotin-cytidine 5′-triphosphate as described (Zhang et al., 2009). Biotinylated transcripts were incubated with cytoplasmic lysates for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway) and analyzed by Western blot analysis.

To assess the association of endogenous HuR with endogenous Lrp6 mRNA, immunoprecipitation (IP) of RNP complexes was performed as described (Xiao et al., 2007; Cui et al., 2012). Twenty million cells were collected per sample, and lysates were used for IP for 4 h at room temperature in the presence of excess (30 μg) IP antibody (IgG, anti-HuR). RNA in IP materials was used in RT followed by PCR and qPCR analysis to detect the presence of Lrp6 and Gapdh mRNAs.

Cell proliferation assays
Cell proliferation was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay kit (Roche, Indianapolis, IN) and flow cytometry analysis (Mosmann et al., 1983; Xiao et al., 2013). For MTT assays, cells were seeded into 96-well plates, and after 48 h, MTT was added to each well. For cell-cycle analysis, isolating and staining of nuclei from cells were performed with a CycleTestPlus DNA Reagent Kit (BD Biosciences, San Jose, CA). After propidium iodide was stoichiometrically bound to the nuclei, the samples were run on a flow cytometer.

Statistics
Results are expressed as the means ± SEM from three to six samples. The significance of the difference between means was determined by Student’s t-test; p < 0.05 was considered significant.

Additional methods
Additional methods, including the procedures for irradiation, historical analysis, assays for in vivo gut permeability, analysis of newly translated protein, and polysome analysis, are given in the Supplementary Methods.

ACKNOWLEDGMENTS
This work was supported by Merit Review Awards to J.Y.W. and J.N.R. from the U.S. Department of Veterans Affairs; National Institutes of Health Grants DK57819, DK61972, and DK68491 to J.Y.W.; Association for International Cancer Research Grant AICR-07-0548 and the European Commission INFLACARE (HEALTH-F2-2009-223151 to D.K.; and the National Institute on Aging-Intra-mural Research Program to M.G. J.Y.W. is a Senior Research Career Scientist, Biomedical Laboratory Research and Development Service, U.S. Department of Veterans Affairs.

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