Long noncoding RNA CRNDE stabilized by hnRNPUL2 accelerates cell proliferation and migration in colorectal carcinoma via activating Ras/MAPK signaling pathways

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Recent studies have furthered our understanding of the function of long noncoding RNAs (lncRNAs) in numerous biological processes, including cancer. This study investigated the expression of a novel lncRNA, colorectal neoplasia differentially expressed (CRNDE), in colorectal carcinoma (CRC) tissues and cells by real-time RT-PCR and in situ hybridization, and its biological function using a series of in vitro and in vivo experiments to determine its potential as a prognostic marker and therapeutic target. CRNDE was found to be upregulated in primary CRC tissues and cells (P < 0.05), and the upregulation of CRNDE expression is a powerful predictor of advanced TNM stage (P < 0.05) and poor prognosis for CRC patients (P = 0.002). The promoting effects of CRNDE on the cell proliferation, cell cycling and metastasis of CRC cells were confirmed both in vitro and in vivo by gain-of-function and loss-of-function experiments. Mechanistically, it was demonstrated that CRNDE could form a functional complex with heterogeneous nuclear ribonucleoprotein U-like 2 protein (hnRNPUL2) and direct the transport of hnRNPUL2 between the nucleus and cytoplasm. hnRNPUL2 that was accumulated in the cytoplasm could interact with CRNDE both physically and functionally, increasing the stability of CRNDE RNA. Moreover, gene expression profile data showed that CRNDE depletion in cells downregulated a series of genes involved in the Ras/mitogen-activated protein kinase signaling pathways. Collectively, these findings provide novel insights into the function and mechanism of lncRNA CRNDE in the pathogenesis of CRC and highlight its potential as a therapeutic target for CRC intervention.

Cell Death and Disease (2017) 8, e2862; doi:10.1038/cddis.2017.258; published online 8 June 2017

Colorectal carcinoma (CRC) is the third leading cause of cancer-related deaths worldwide, and its incidence is on the rise.1 Although it is well known that multiple known carcinoma and varying genetic backgrounds are involved in the tumorigenesis and progression of CRC, the detailed interactions and regulatory mechanisms of key pathways implicated in the progression of the disease are still obscure. Therefore, there is an urgent need for the identification of a reliable molecule that is involved in the progression of CRC and of novel targets for effective intervention.

Recent improvements have revealed that a substantial portion of the human genome can be transcribed into many short or long noncoding RNAs (lncRNAs).2,3 LncRNAs are transcripts longer than 200 bp that do not have any apparent protein-coding ability. Although to data, only a small number of functional lncRNAs have been well characterized, accumulating data suggest that lncRNAs are powerful transcriptional and post-transcriptional regulators to modulate downstream target genes and participate in diverse physiological and pathological processes.4 The aberrant expression of lncRNAs has been demonstrated in multiple malignancies,5–10 including CRC, providing new insights into the pathogenesis of cancer. However, the potential role of lncRNAs in CRC pathogenesis and progression remains obscure.

The novel lncRNA, colorectal neoplasia differentially expressed (CRNDE), was originally discovered as an upregulated gene in colorectal adenomas and cancers, whereas there is little to no expression in normal colon epithelia.11 At least 10 splice variants of CRNDE have been identified. The expression level of CRNDE-h transcript was upregulated in the plasma of CRC patients, and the expression levels of this transcript alone have shown a sensitivity of 87% and specificity of 93% for predicting the presence of CRC.11 In addition to CRC, CRNDE overexpression has been observed in many other solid tumors and lymphocytic leukemias.12 In glioma, CRNDE is the most highly expressed lncRNA,12,13 and it has been shown to affect the malignant biological characteristics of glioma stem cells.14 In ovarian cancer, elevated levels of CRNDE were found to be a negative prognostic factor, increasing the risk of death and recurrence in ovarian cancer patients treated with platinum compounds and taxanes.15 Very recently, a study has shown that increased expression of CRNDE is correlated with a poor prognosis in CRC.16 Collectively, the current evidence suggests that CRNDE overexpression appears to have a key role in tumorigenesis. However, the precise function and mechanism behind CRNDE overexpression in CRC and the

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Received 10.2.17; revised 25.4.17; accepted 04.5.17; Edited by A Oberst
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downstream molecules associated with its action are largely unknown.

In this study, we demonstrated that increased CRNDE expression is a characteristic molecular change in CRC and investigated the effects of aberrant CRNDE expression on the cellular biological behavior of CRC cells. We further present evidence of CRNDE interacting with heterogeneous nuclear ribonucleoprotein U-like 2 protein (hnRNPUL2) protein and activating Ras/mitogen-activated protein kinase (MAPK) signaling pathways in CRC cells. Our findings provide novel insights into the function and mechanisms of CRNDE in CRC pathogenesis and a potential therapeutic target for CRC intervention.

Results

CRNDE is upregulated in CRC tissues and cell lines. The expression levels of CRNDE were detected by real-time RT-PCR in a panel of CRC cell lines and 30 paired CRC and adjacent non-cancerous mucosa tissues. An increase in CRNDE expression was seen in CRC tissues compared with paired non-cancerous tissues (P = 0.038, Figure 1A). Compared with a colon mucosa epithelial cell line (NCM460), CRNDE expression was higher in seven out of eight CRC cell lines, the exception being LS174T (Figure 1B).

High CRNDE expression is associated with poor prognosis in CRC patients. We assessed CRNDE expression in CRC tissues by in situ hybridization (ISH). We observed that CRNDE was mainly expressed in the cytoplasm and that it was expressed in 89.64% (225 of 251) of all CRC samples, but in only 19.53% (25 of 128) of adjacent non-cancerous tissues. The levels of CRNDE were significantly upregulated in CRC tissues (P < 0.001, Figure 1C). From the clinical data, it was found that high levels of CRNDE expression were associated with T stage, N stage and M stage (Figure 1D and Table 1). Furthermore, Kaplan–Meier analysis revealed that higher CRNDE expression in CRC tissues was significantly correlated with reduced overall survival in CRC patients (P = 0.002. log-rank test = 7.294; Figure 1E). In addition, multivariate survival analysis was performed including the parameters of gender, age, tumor size, tumor differentiation, T stage, N stage, M stage and CRNDE expression level. From this analysis, we identified CRNDE expression level as an independent prognostic factor of disease outcome in CRC patients (Table 2).

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**Figure 1**   CRNDE expression is upregulated in CRC and could be an independent prognostic factor for the prediction of the overall survival of CRC patients. (A) Expression levels of CRNDE in paired CRC and adjacent non-cancerous tissues. (B) Expression levels of CRNDE in CRC and colon mucosa epithelial (NCM460) cell lines. (C) Expression analysis of CRNDE in normal colorectal mucosa and CRC tissues by ISH. (a) Negative expression of CRNDE in normal colorectal mucosa. (b) High expression of CRNDE in a tumor tissue sample and weak expression of CRNDE in its normal mucosal counterpart were observed in one filed of a tissue sample from a single patient. (c) High expression of CRNDE in CRC tissue. Scale bars are shown in the lower right corner of each picture. (d) Graphical illustration of statistical CRNDE distribution in CRC patients. (E) Kaplan–Meier analysis of overall survival in all patients with CRC according to CRNDE expression. *P < 0.05
Knockdown of CRNDE inhibits CRC cell proliferation, invasion and migration in vitro. To explore the role of CRNDE in CRC oncogenesis, we first downregulated CRNDE expression by cloning CRNDE short-hairpin RNA (shRNA) into lentivirus to establish loss-of-function models in CRC cell lines including in DLD1 and HCT116 cells. Real-time RT-PCR analysis revealed that CRNDE was strikingly downregulated in the RNAi group compared with the control group (P<0.001, Figure 2a). To clarify whether there are off-target binding sites, we analyzed the knockdown efficiency of CRNDE shRNA in CRC cells with ectopic overexpression of CRNDE. CRNDE levels were significantly reduced again after the CRNDE-overexpressing CRC cells were transfected with anti-CRNDE siRNAs compared with the control group (Supplementary Figure S1). The Cell Counting Kit-8 (CCK-8) assay was used to explore the biological effects of CRNDE on cell proliferation and revealed that the downregulation of CRNDE could markedly inhibit CRC cell growth in vitro (P=0.001, Figure 2b). Similarly, the capacity to form colonies in the CRNDE-depleted cells was suppressed compared with the control group (DLD1: P=0.002 and HCT116: P=0.022, Figure 2c). Moreover, cell cycle progression and apoptosis detection were performed by flow cytometry, which revealed that the depletion of CRNDE could result in an increased percentage of G1-phase cells in two of the CRC cell lines (DLD1: P=0.020 and HCT116: P=0.018, Figure 2d). It was observed that the decrease of CRNDE expression could induce cell apoptosis and enhance cleavage/activation of caspase-3 and -9 in HCT116 cells (P=0.007, Figure 2e). However, the proportion of apoptotic cells remained similar between DLD1 with CRNDE-knockdown and control cells (P=0.394, Supplementary Figure S2).

Moreover, we observed the effect of CRNDE on the invasion and migration capacity of CRC cells via a Matrigel invasion assay and wound-healing assay. Matrigel invasion analysis demonstrated that the knockdown of CRNDE in CRC cells strongly reduced cell invasiveness (DLD1: P<0.001 and HCT116: P=0.007, Figure 2f). The wound-healing assay also illustrated that downregulation of CRNDE expression reduced the cell migration capacity (DLD1: P=0.0097 and HCT116: P=0.030, Figure 2g). These data suggest that CRNDE is necessary for cell invasion and migration.

Knockdown of CRNDE reduces human CRC cell proliferation and metastasis in mice in vivo. We subcutaneously injected CRC cells with stable CRNDE downregulation or control cells into nude mice for xenotransplantation. Consistent with the in vitro results, mice injected with CRNDE-depleted DLD1 cells showed significantly decreased tumor growth compared with those injected with control cells (Figures 3a and b).

Next, we established a peripheral intravascular implanted metastatic model by injecting DLD1 cells with downregulated CRNDE expression or negative control cells into nude mice through the tail vein. At 4 weeks after injection, the mice were killed, and the lungs were subjected to histological analysis. The results showed that CRNDE knockdown decreased the

**Table 1** Correlation between the clinicopathological features and expression of CRNDE

| Characteristics | n | Low (%) | High (%) | P-value |
|-----------------|---|---------|----------|---------|
| Gender          |   |         |          |         |
| Male            | 150 | 77 (51.33) | 73 (48.67) | 0.074   |
| Female          | 101 | 54 (53.47) | 47 (46.53) |         |
| Age (years)     |   |         |          |         |
| < 50            | 113 | 66 (58.41) | 47 (41.59) | 0.74    |
| ≥ 50            | 138 | 65 (47.10) | 73 (52.90) |         |
| Tumor size (cm in diameter) |   |         |          |         |
| < 5             | 100 | 57 (57.00) | 43 (43.00) | 0.215   |
| ≥ 5             | 151 | 74 (49.01) | 77 (50.99) |         |
| Tumor differentiation | |       |          |         |
| Good            | 96  | 45 (46.88) | 51 (53.12) | 0.058   |
| Moderate        | 99  | 49 (49.49) | 50 (50.51) |         |
| Poor            | 56  | 37 (66.07) | 19 (33.93) |         |
| T stage         |   |         |          |         |
| 1–2             | 59  | 35 (59.32) | 24 (40.68) | 0.027   |
| 3               | 186 | 96 (51.61) | 90 (48.39) |         |
| 4               | 6   | 0 (0.00)  | 6 (100.00) |         |
| N stage         |   |         |          |         |
| 0               | 156 | 89 (57.05) | 67 (42.95) | 0.048   |
| 1–2             | 96  | 42 (44.21) | 53 (55.79) |         |
| M stage         |   |         |          |         |
| 0               | 240 | 129 (53.75) | 111 (46.25) | 0.021   |
| 1               | 11  | 2 (18.18)  | 9 (81.82)  |         |

Abbreviations: CRNDE, colorectal neoplasia differentially expressed.

**Table 2** Summary of overall survival analyses by univariate and multivariate Cox regression analysis

| Variables     | Univariate analysis | Multivariate analysis |
|---------------|---------------------|-----------------------|
|               | P-value | HR     | CI (95%)    | P-value | HR     | CI (95%)    |
| Gender        | 0.483   | 1.169  | 0.756–1.808 |         |        |          |
| Age           | 0.686   | 1.094  | 0.707–1.693 |         |        |          |
| Tumor size    | 0.023   | 0.576  | 0.358–0.927 |         |        |          |
| Tumor differentiation | 0.021 | 1.427  | 1.055–1.930 |         |        |          |
| T stage       | 0.001   | 2.33   | 1.391–3.904 |         |        |          |
| N stage       | 0.001   | 2.118  | 1.372–3.269 |         |        |          |
| M stage       | <0.001  | 7.451  | 3.779–14.690|         |        |          |
| CRNDE expression | 0.002 | 2.024  | 1.291–3.173 |         |        |          |

Abbreviations: CI, confidence interval; CRNDE, colorectal neoplasia differentially expressed; HR, hazard ratio.
number of definite pulmonary colonization sites (5 out of 20 mice) compared with the control group (10 out of 20 mice) \((P = 0.046, \text{Figures 3c and d})\). Moreover, compared with mice injected with control cells, the number of pulmonary tumor colonies per microscopic view in the CRNDE-knockdown cell-injected mice was significantly decreased \((P = 0.036, \text{Figure 3d})\). Taken together, these data indicate an important role for CRNDE in promoting tumor metastasis in vivo, which is supported by in vitro results.

Overexpression of CRNDE promotes CRC cell growth, invasion and migration in vitro. We also established gain-of-function models to investigate the changes in the biological behavior of SW480 and LS174T cells following CRNDE overexpression. Real-time RT-PCR analysis revealed that CRNDE was significantly upregulated in both SW480 and LS174T cells that infected with lentivirus carrying the CRNDE-h gene compared with the control group (Supplementary Figure S1). As shown in Figure 4a,
CCK-8 assay indicated when CRNDE expression was enhancing, the proliferation rate of CRC cells was significantly increased, compared with the mock cells ($P < 0.001$). Similarly, compared with the control cell lines, the capacity to form colonies was increased in the CRNDE-overexpressing cells (SW480: $P = 0.027$ and LS174T: $P = 0.031$, Figure 4b). Flow cytometry also revealed that the overexpression of CRNDE increased the proportion of G2-phase cells (SW480: $P = 0.006$ and LS174T: $P = 0.018$, Figure 4c). However, the proportion of apoptotic cells remained similar between CRNDE-overexpressing and control cells ($P > 0.05$, Supplementary Figure S2). Meanwhile, we also observed that ectopic overexpression of CRNDE increased the capacity of invasion ($P < 0.001$, Figure 4d) and migration in both SW480 and LS174T cells (SW480: $P = 0.004$ and LS174T: $P = 0.001$, Figure 4e).

**CRNDE binds to hnRNPUL2 protein and directs its localization.** To identify the proteins that bind to CRNDE, we performed an RNA pull-down experiment in DLD1 cells. We separated the RNA-associated proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), excised the bands specific to CRNDE and subjected them to mass spectrometry (Figure 5a). Interestingly, hnRNPUL2 was the most abundant protein among all of the proteins identified by mass spectrometry (Table 3), suggesting an interaction between hnRNPUL2 and CRNDE. Western blotting analysis also was carried out to detect hnRNPUL2 (Figure 5b). RNA immunoprecipitation (RIP) assay with an antibody against hnRNPUL2 was performed to further validate the interaction between CRNDE and hnRNPUL2. Significant CRNDE RNA enrichment was observed using the hnRNPUL2 antibody compared with using a nonspecific antibody (IgG control) ($P = 0.004$, Figure 5c). These analyses confirmed that CRNDE physically associates with hnRNPUL2 in vitro.

Furthermore, we measured and compared the levels of hnRNPUL2 expression in CRC cells with CRNDE overexpression or CRNDE depletion. However, there was no significant difference in either hnRNPUL2 mRNA or total protein levels in SW480 cells with CRNDE upregulation or DLD1 cells with CRNDE downregulation, compared with that in the control cells (Figure 5d). Although all of the hnRNP proteins are present in the nucleus, some seem to shuttle between the nucleus and the cytoplasm. It has been reported that lncRNAs can direct the localization of target proteins within cellular compartments. Therefore, we speculated that CRNDE might influence the localization of hnRNPUL2 in CRC cells. To clarify our hypothesis, nuclear and cytoplasmic protein fractions were prepared from CRC
It was found that the overexpression of CRNDE induced a subcellular relocalization of hnRNPUL2 and increased the amounts of hnRNPUL2 that were seen in the cytoplasm. Conversely, the downregulation of CRNDE appeared to reduce the cytoplasmic levels of hnRNPUL2 expression in DLD1 and HCT116 cells (Figures 5e and f). These results suggested that CRNDE could bind to hnRNPUL2 protein and direct its transition between the nucleus and the cytoplasm.

Cytoplasm-accumulated hnRNPUL2 stabilizes CRNDE RNA. Given the earlier results demonstrating that hnRNPUL2 proteins shuttle between the nucleus and the cytoplasm, it is also possible that they have cytoplasmic functions. Previous studies have implicated hnRNPs in the regulation of mRNA stability.\textsuperscript{18, 19} Therefore, we examined the effects of cytoplasmic hnRNPUL2 protein on the stability of CRNDE. CRNDE-overexpressing SW480 cells were first transfected with hnRNPUL2-siRNA or control siRNA and then treated with 1 μg/ml actinomycin D over a 6-h period to block new RNA synthesis. Then, CRNDE expression was assessed. As shown in Figures 5g and h, the siRNA-mediated hnRNPUL2 depletion was found to reduce CRNDE stability and CRNDE expression levels following the cytoplasmic retention of hnRNPUL2 that was induced by CRNDE overexpression; meanwhile, this reduction of CRNDE levels was not observed in no CRNDE-overexpressing cells compared with its corresponding controls, implying that an increased content of cytoplasmic hnRNPUL2 following CRNDE overexpression leads to an increase in the CRNDE expression level. These results collectively suggest that CRNDE overexpression induces hnRNPUL2 to accumulate in the cytoplasm, which allows for its interaction with CRNDE, leading to an increase in the stability of CRNDE expression. Thus, we concluded that the interaction between CRNDE and

Figure 4 Overexpression of CRNDE promotes cell proliferation, cell cycling, invasion and migration in CRC cells in vitro. (a) Overexpression of CRNDE induced significantly higher growth rates in CRC cells. (b) Overexpression of CRNDE increased the capacity to form colonies in CRC cells. (c) Overexpression of CRNDE induced a significant increase in cells at G2-phase relative to mock cells. (d) and (e) Invasion/migration assays using Matrigel Transwell and wound-healing assays for CRC cells. CRNDE overexpression promoted the invasion and migration of CRC cells. The experiments were performed in triplicate; the data are expressed as the mean ± S.D. *P < 0.05, **P < 0.01 and *** P < 0.001.
hnRNPUL2 is not only a physical one but also a functional one.

**CRNDE upregulates the expression of Ras/MAPK signaling genes.** To identify the genes targeted by CRNDE and explore the mechanism of action of CRNDE on CRC tumorigenesis, gene expression profiling was performed on CRNDE-depleted DLD1 cells and control cells. Many differentially expressed genes (>2-fold; <0.05 false discovery rate) were identified (GSE89985). KEGG pathway analysis indicated that Ras signaling pathway was the main pathway associated with the downregulation of CRNDE (Figure 6a). Notably, many pathways known to be associated with cancer, including the MAPK signaling pathway, were found to be deregulated in CRNDE-depleted DLD1 cells. The results showed that CRNDE affected the expression of a group of functional genes that has similar biological effects in cancer (Figure 6b). Twenty-one Ras signaling genes whose expressions were suppressed in the CRNDE-depleted cells were verified in the CRNDE-overexpressing CRC cells and...
control groups by real-time RT-PCR analysis. All genes showed expression trends that were consistent with the gene chip results (Figure 6c).

Discussion

It is well known that IncRNAs are an important player in cancer biology, typically causing the aberrant expression of gene products that contribute to the progression of a number of human cancers. A growing body of evidence has indicated that IncRNAs contribute to the progression of CRC. For example, IncRNA CCAL regulates CRC progression by activating the Wnt/β-catenin signaling pathway via the suppression of activator protein 2a. The overexpression of CCAL can be used as an indicator of poor survival and can predict the response to adjuvant chemotherapy in CRC patients. CRNDE is a lncRNA that was originally identified in CRC. However, the function and underlying mechanisms of CRNDE in influencing CRC progression are still largely unknown. In the present study, we studied a large cohort of CRC patients and determined that high-expression levels of CRNDE were significantly associated with aggressive stages and cancer-related deaths of CRC patients. This association was independent of other clinical covariates, indicating that CRNDE depletion may decrease tumor growth and improve survival in CRC patients. Further studies are required to establish the clinical availability of CRNDE inhibition as a novel therapeutic strategy.

We investigated the mechanisms of action by which CRNDE exerts its modulatory effect on malignant CRC phenotypes. LncRNAs enriched in the cytoplasm typically participate in post-transcriptional regulation by interacting with microRNA or mRNA. A previous study reported that CRNDE decreased the expression of XIAP and PAK7 by binding to and inhibiting miR-186, subsequently affecting the malignant biological characteristics of glioma stem cells. However, we assume that this is not the primary function of CRNDE. LncRNAs have been reported to exert their function by various mechanisms, including interacting with proteins to modulate protein function, regulating protein–protein interactions and directing localization within cellular compartments. These interactions are central to determining the functional effects of IncRNA. An RNA pull-down assay revealed that CRNDE could combine with the hnRNPU2, increasing the cytoplasmic translocation of hnRNPU2 protein.

As RNA-binding proteins, hnRNP have been implicated in diverse cellular processes, such as modulating splicing, miRNA transport, miRNA maturation and mRNA stability. It has been shown that hnRNPU2 could interact with NBS1, a subunit of the DNA double-strand break (DSB) sensor complex MRE11-RAD50-NBS1, and be recruited to sites of DNA damage to stimulate DNA-end resection and promote DSB repair. However, the functional roles of hnRNPU2 remain largely obscure.

The present study identifies hnRNPU2 as an important player in regulating CRNDE expression. The majority of hnRNPU2 is retained in the nucleus. Only a small fraction of hnRNPU2 is in the cytoplasm. Our study suggested that...
CRNDE overexpression led to an increase in hnRNPUL2 accumulation in the cytoplasm and that this cytoplasmic
hnRNPUL2 was responsible for the interaction with CRNDE. Increased cytoplasmic accumulation of hnRNPUL2 was
accompanied by increased expression of CRNDE as a result of the increased stability of CRNDE by hnRNPUL2-bound
CRNDE in the cytoplasm. Thus, we concluded that there exists a feedback loop between CRNDE and hnRNPUL2.

Recent studies have shown that the hnRNP family interacts with multiple lncRNAs. Huarte et al. identified that the
p53-inducible IncRNA-p21 bound to hnRNK to mediate gene repression in response to DNA damage. Similarly, IncRNA
CASC11 has also been reported to interact with hnRNPK and activate the Wnt/β-catenin pathway to promote growth and
metastasis in CRC. These findings make it tempt to conclude that hnRNK proteins are widespread mediators of
IncRNA function. The present study provides an additional mechanism by which hnRNPUL2 may affect IncRNA stability.
Thus, the present findings supplement and extend the current knowledge on the functional role of RNA–protein complexes.

Another major finding of the present study is the identification of downstream molecules associated with CRNDE
function. The data obtained from the mRNA expression profile of CRNDE-depleted CRC cells indicate that CRNDE has a
broad regulatory function involving the Ras and MAPK pathways. Activation of Ras signaling is arguably the most
common biomolecular event in human cancer. Activated Ras activates a series of kinase cascades, including RAF,
MEK and MAPK. These have functions as molecular switches for signaling pathways regulating cell proliferation, survival,
migration, differentiation and cytoskeletal dynamism. However, in reality, many other proteins are also involved in this
pathway, including EGFR, GRB2, and VEGF and the PDGF family. A number of researchers have demonstrated that upon stimulation with growth factors, or other inputs, the Ras/MAPK system can promote cell proliferation, differentiation and/or migration. Of note, many of the Ras/MAPK signaling genes induced by CRNDE are known positive regulators of CRC tumorigenesis, including Tiam1, GRB2, and RIN1. These data collectively suggest that CRNDE is a widespread mediator of genes involved in the progression of CRC. Future work will be need to elucidate the mechanism by which CRNDE regulates the expression of those genes and to further delineate the network controlled by CRNDE in CRC progression.

In summary, this study shows that increased CRNDE expression is a characteristic molecular change in CRC and
that the upregulation of CRNDE expression is a powerful predictor of advanced TNM stage and poor prognosis for CRC
cancer. The elevation of CRNDE expression promotes cell proliferation and metastasis, implying that CRNDE inhibition
can be a novel therapeutic modality for CRC patients. We suggest that cytoplasmic hnRNPUL2 is an important mediator
that induces CRNDE overexpression via increasing the
stability of CRNDE, followed by activating the Ras/MAPK signaling pathways. Our findings provide novel insights into the functions and mechanisms of IncRNA CRNDE in the pathogenesis of CRC and highlight its potential as a therapeutic target for CRC intervention.

Materials and Methods

Ethics statement. The use of tissues for this study has been approved by the ethics committee of Nanfang Hospital, Southern Medical University (Guangzhou, China). All of the patients provided signed, informed consent before the use of these clinical materials for research purposes. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Southern Medical University.

Tissue specimens and cell culture. All CRC specimens were obtained from patients who had been diagnosed with primary CRC and had subsequently undergone elective surgery in Nanfang Hospital, Southern Medical University. Freshly frozen tumor samples from 30 CRC patients were selected for real-time RT-PCR. Formalin-fixed tumor tissue samples, comprising of 251 CRC tumor tissues and 128 adjacent non-tumors tissues, were used for IHC analysis. A comprehensive set of clinicopathological data was recorded. Complete follow-up, which ranged from 1 to 117 months, was achieved for all patients, and the median survival duration was 57 months.

The human CRC cell lines DLD1, HCT116, SW480, SW620, LoVo, LoVo, LS174T and HT29 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A subclone named M5 with enhanced metastatic abilities in the liver was isolated by the in vivo selection of SW480 cells in our laboratory.46 All CRC cell lines were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) at a humidity of 5% CO2 at 37 °C.

ISH and evaluation of CRNDE staining. ISH was performed according to the manufacturer's protocol (Boster Bio-Engineering Company, Wuhan, China). Briefly, 4-μm-thick paraaffin-embedded sections were deparaffinized with xylene and rehydrated with dilute ethanol of reagent grade. The samples were digested with 20 μg/ml Proteinase K, fixed in 4% paraformaldehyde, hybridized with the 5'-digoxigenin-labeled probe of CRNDE with a sequence of 5′-CTTGTGTGACGGGAG-GGG-3′ at 55 °C overnight, and subsequently incubated for 30 min at 4 °C with HRP. Diaminobenzidine was used to develop the stain with a color reaction.

The ISH-stained tissue sections were reviewed and scored separately by two blinded pathologists. Scores were determined using a relatively simple, reproducible scoring method based on both the intensity and proportion of CRNDE-positive cells.46 The staining intensity was scored on a scale of 0–3, as follows: negative (no staining, 0), weak (1), medium (2) or strong (3). The extent of the staining was defined as the percentage of the positive stained areas of tumor cells or normal epithelial cells in relation to the whole tumor area or the entire section of the normal samples, and it was scored on a scale of 0–4 as follows: 0% (0); 1–25% (1); 26–50% (2); 51–75% (3); and 76–100% (4). The sum of the staining-intensity and staining-extent scores was used as the final staining score for CRNDE (on a scale of 0–7). A final staining score of ≥3 was considered to denote high-expression of CRNDE.

Construction of cell lines with stably downregulated CRNDE. Three shRNA sequences specially targeting CRNDE were designed and synthesized (Supplementary Information), and cloned into a pGPU6/GFP/Neo-shRNA vector (GenePharma, Shanghai, China). The most effective shRNA sequence in achieving knockdown of CRNDE was successfully subcloned into a pcDNA3.1 vector. CRC cell lines with ectopic expression of CRNDE were achieved by pcDNA3.1-CRNDE transfection and CRC cells transfected with empty pcDNA vector were used as control (mock). The expression levels of CRNDE were detected by real-time RT-PCR.

RNA isolation and real-time PCR. Total RNA was extracted using TRIzol Reagent (Takara, Dalian, China). RNA fractionation into cytoplasmic and nuclear lysates was carried out using Protein and RNA Isolation System Kit (Ambion by life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara), Real-time RT-PCR was performed to detect the expression of CRNDE using One-Step SYBR PrimeScript RT-PCR Kit (Takara). The results were normalized to the expression of β-actin. The assay was performed in triplicate for each case to allow for the assessment of technical variability. The primer sequences used for PCR are listed in Supplementary Information.

Cell proliferation assay and colony formation assay. Cells were seeded in 96-well plates at 2 × 103 cells well per well. Cell proliferation was evaluated using CCK-8 (Dojindo, Rockville, MD, USA) according to the manufacturer's instructions. For the colony formation assay, the cells were plated in 6-well plates at 2 × 103 cells per well and maintained in RPMI 1640 containing 10% FBS for 2 weeks. After 2 weeks, the cells were washed twice with PBS, fixed with methanol and stained with Giemsa. The number of colonies was counted under a microscope. All experiments were performed in triplicate.

Wound-healing and invasion assays. Cell migration was assessed by measuring the movement of cells into a scraped, acellular area that was prepared as described previously.46 The spread extent of wound closure was observed after 0 and 48 h, respectively. Migration was quantified by counting the total number of cells that migrated toward the original wound field. For the invasion assay, Matrigel-coated chambers (BD Biosciences, San José, CA, USA) containing 8-μm pores were used. A total of 2 × 104 concentration cells were seeded into the upper chambers (coated in Matrigel) in serum-free medium. The lower chamber of the Transwell was filled with culture media containing 10% FBS as a chemo-attractant.

All the chambers were incubated at 37 °C for 48 h, non-invaded cells on the top of the Transwell were scraped off with a cotton swab. Successfully translocated cells were fixed with 10% formalin. Then, they were stained with 0.1% crystal violet for 30 min and counted under a light microscope. All experiments were performed in triplicate.

In vivo functional assays in mouse models. Balbc/Cn nu/nu nude mice were obtained from the Laboratory Animal Center of Southern Medical University. For in vivo tumorigenicity, CRCNDE-depleted DLD1 cells and control cells were typsinized, counted and reseeded in sterile PBS. A total of 5 × 106 CRNDE-depleted DLD1 cells or control cells were subcutaneously injected into the right and left bilateral upper limbs of mice (4–6 weeks of age), respectively. The mice were then monitored for tumor volume and overall health. The size of the tumor was determined by caliper measurement of the tumor mass. Tumor volume was calculated according to the formula 0.5 × length × width². Each experimental group contained five mice.

For developing the in vivo metastatic model, mice were injected intravenously via the lateral tail vein in 5 × 106 cells. After 4 weeks of monitoring, mice were killed by cervical dislocation. The lungs were removed by dissection away from adjacent organs, and fixed using 10% neutral-buffered formalin. Subsequently, the consecutive tissue sections were obtained and stained with hematoxylin-eosin (H&E) to observe the metastatic nodules of the lungs under the microscope.

Western blot analysis. Protein lysates from cells were separated by 10% SDS-PAGE, transferred to 0.22-μm NC membranes (Sigma, Shanghai, China) and incubated with anti-human (h)RNPUL2 rabbit polyclonal antibody (1:2000; Abcam, Cambridge, UK) or rabbit polyclonal antibodies against caspase-9, cleaved caspase-9, caspase-3 and cleaved caspase-3 (1:500; CST, Danvers, MA, USA). The band intensity was measured by densitometry using the Quantity One Software (Bio-Rad, West Berkeley, CA, USA). The protein levels were normalized with that of window.
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10. Acknowledgements. The National Basic Research Program of China (973 Program, 2015CB554002), the National Natural Science Foundation of China (81472318) and the National Natural Science Foundation of Guangdong Province (2014A030313300) supported this work.

11. Immunofluorescence analysis. Different CRC cell lines were cultured and fixed on 12 x 12 mm glass slides. After incubating with antibodies specific for hnRNPU2c (Abcam) and then with goat anti-rat IgG (Alexa Fluor 594; Invitrogen, Carlsbad, CA, USA), the slides were mounted by adding DAPI-Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and examined with an Olympus V100 confocal laser scanning biological microscope (Olympus Corporation, Tokyo, Japan).

12. RNA pull-down assays. RNA pull-down assays were performed as described previously. Briefly, CRNDE-h and its antisense RNA were in vitro transcribed from the vector pcDNA3.1-CRDNE. RNAs were biotin-labeled with the Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN, USA) and in vitro transcribed using the T7/SP6 RNA polymerase (MEGAscript Kits; Ambion). The protein extracted from DLD1 cells was mixed with biotinylated RNAs, incubated with magnetic beads (Life Technologies, Carlsbad, CA, USA) and washed. The retrieved proteins were resolved by SDS-PAGE, and then silver-stained. Specific bands were excised and analyzed by mass spectrometry.

13. RNA immunoprecipitation. RIP assays were performed according to the instructions provided in the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Briefly, cells were crosslinked with 1% (vol/vol) formaldehyde and suspended in lysis buffer containing a protease inhibitor cocktail and an RNase inhibitor. Magnetic beads were preincubated with an anti-rabbit IgG and an RNase inhibitor. Magnetic beads (Life Technologies, Carlsbad, CA, USA) and washed. The retrieved RNAs were biotin-labeled with the Agilent Oligo Microarray (4x44K; Agilent Technologies, Santa Clara, CA, USA). The labeled cRNA was then hybridized onto the Human Genome Oligo Microarray (4x44K; Agilent Technologies, Santa Clara, CA, USA). The arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies). The Agilent Feature Extraction software (version 11.0.1.1, Santa Clara, CA, USA) was used to analyze the acquired array images. Quantize (Agilent Technologies). The putative oncogene, CRNDE, is a negative prognostic factor in ovarian cancer patients. Cancer Discov 2015; 5: 2353–2355.
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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)