A HIC-5- and KLF4-dependent Mechanism Transactivates p21\(^{\text{Cip1}}\) in Response to Anchorage Loss*\(^{\text{S}}\)

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Kazunori Mori, Hiroyuki Hamanaka, Yukiko Oshima, Yuri Araki, Fumihiro Ishikawa, Kiyoshi Nose, and Motoko Shibanuma

From the Department of Molecular Biology, Division of Cancer Cell Biology, Showa University School of Pharmacy, Tokyo 142-8555, Japan

Background: Anchorage dependence of cell growth remains incompletely understood.

Results: A cyclin-dependent kinase inhibitor (p21\(^{\text{Cip1}}\)) is transcriptionally up-regulated on anchorage loss, depending on Kruppel-like factor 4 (KLF4) and a molecular scaffold of hydrogen peroxide-inducible clone-5 (HIC-5).

Conclusion: On anchorage loss, HIC-5 localizes at the nuclear matrix and promotes KLF4 tethering to DNA.

Significance: A novel mechanism regulating gene expression in a detachment-dependent manner has emerged.

Anchorage loss elicits a set of responses in cells, such as transcriptional changes, in order to prevent inappropriate cell growth in ectopic environments. However, the mechanisms underlying these responses are poorly understood. In this study, we investigated the transcriptional up-regulation of cyclin-dependent kinase inhibitor p21\(^{\text{Cip1}}\) during anchorage loss, which is important for cell cycle arrest of nonadherent cells in the G1 phase. Up-regulation was mediated by an upstream element, designated as the detachment-responsive element (DRE), that contained Kruppel-like factor 4 (KLF4) and runt-related transcription factor 1 (RUNX1) recognition sites; both of these together were necessary for transactivation, as individually they were insufficient. RNAi experiments revealed that KLF4 and a multidomain adaptor protein, hydrogen peroxide-inducible clone 5 (HIC-5), were critically involved in DRE transactivation. The role of HIC-5 in this mechanism was to tether KLF4 to DNA sites in response to cellular detachment. In addition, further analysis suggested that oligomerization and subsequent nuclear matrix localization of HIC-5, which was accelerated spontaneously in cells during anchorage loss, was assumed to potentiate the scaffolding function of HIC-5 in the nucleus and consequently regulate p21\(^{\text{Cip1}}\) transcription in a manner responding to anchorage loss. At the RUNX1 site, a LIM-only protein, CRP2, imposed negative regulation on transcription, which appeared to be removed by anchorage loss and contributed to increased transcriptional activity of DRE together with regulation at the KLF4 sites. In conclusion, this study revealed a novel transcriptional mechanism that regulated gene expression in a detachment-dependent manner, thereby contributing to anchorage-dependent cell growth.

Physical and functional cellular adhesion to the extracellular matrix (ECM)\(^{2}\) is indispensable to multicellular organisms from embryonic stages throughout maturity (1, 2). In metazoans, cellular adhesion to ECM is mediated by integrins, a class of heterodimeric transmembrane receptors, which on encounter with a ligand, transmit intracellular signals activating numerous downstream classical kinase- and GTPase-mediated pathways (3). Interestingly, the pathways activated by integrin are largely the same as those activated by growth factor and cytokine receptors, and these signals potentiate each other downstream, as observed in the synergistic activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase cascade (4–6).

In numerous cellular activities, cooperation between growth factor receptor kinase and integrin signaling is observed, which is manifested as dependence of activities on cellular adhesion to ECM along with growth factors. A typical example is cell cycle progression where various events from mid- to late-G1 phase culminating in up-regulation of G1-phase cyclin-dependent kinase (CDK) activity require both integrin and growth factor signaling (4–7). In mesenchymal cells, in particular, deprivation of ECM anchorage arrests the cell cycle at the G1-phase, which has led to the concept of anchorage-dependent cell growth and is usually interpreted as a requirement of adhesion for complete activation of G1-phase CDKs, although the detailed mechanisms are not fully understood. For multicellular organisms, the anchorage dependence of cell growth is critical because it prevents promiscuous reattachment and inappropriate proliferation of nonadherent cells in ectopic environments.

Hydrogen peroxide-inducible clone-5 (HIC-5) is a multidomain protein comprising four Leu- and Asp-rich LD and LIM domains (named after the three transcriptional factors Lin-11,
Is1-1, and Mec-3) that serve as molecular adaptors in various cellular activities, including integrin signaling at focal adhesions (8, 9) and nuclear transcriptional activities (10). In addition, with a shuttling ability between the two compartments, HIC-5 is capable of coupling cell adhesion with nuclear activities (11). For example, we recently characterized a fail-safe system organized by HIC-5 for anchorage-dependent cell growth (12). The essence of the system was localization of cyclin D1 to the nuclei of only adherent cells and its exclusion from the nucleus on anchorage loss, which is crucial to prevent cell proliferation under nonadherent conditions. This is achieved by adhesion-dependent shuttling of HIC-5, which is regulated by the CRM-dependent nuclear export mechanism (11) and competitively localizes cyclin D1 to the nuclei of adherent cells. On anchorage loss, shutting is stopped, and cyclin D1 is consequently exported outside the nucleus instead of HIC-5 (12).

In the present study, we addressed another HIC-5-dependent mechanism contributing to growth arrest under nonadherent conditions. The mechanism transcriptionally induced a CDK inhibitor (CKI), p21Cip1, in response to disruption of cell-ECM interactions. In this mechanism, HIC-5 played a crucial role in recruitment and/or retention of Kruppel-like factor 4 (KLF4), a transcription factor essential for transactivation, to DNA sites in a detachment-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—C3H10T1/2 cells, primary mouse embryo fibroblasts (MEFs), and normal human diploid fibroblasts (TIG-7) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum as reported previously (12). TIG-7 cells (JCRB0511) were obtained from the Japanese Collection of Research Bioresources Bank (Osaka, Japan) and used between 33 and 39 passages.

The cells were cultured under adherent (adhesion; Adh) and nonadherent (suspension; Sus) conditions in essentially the same manner as described previously (12). In brief, the cells were detached from a culture dish by trypsinization, divided in half, reseeded onto poly (2-hydroxyethyl methacrylate) (Sigma)-coated (0.8 mg/cm²) (Sus) and noncoated (Adh) dishes, and analyzed simultaneously.

**Reagents**—Blasticidin was obtained from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan), and puromycin and doxycycline hyclate (Dox) were purchased from Sigma. Small interfering RNA (siRNA) oligonucleotides used in the present study are listed in the supplemental information.

**Expression Vectors**—Short hairpin RNA (shRNA) was expressed using CS-RfA-ETBsd (Tet-On), CS-RfA-ErTBsd (Tet-Off), and CS-RfA-EB (constitutive) lentiviral vectors (13). The shRNA target sequence information is provided in the supplemental information.

Retrovirus expression vectors were based on pMXs-IP (14). FLAG-tagged HIC-5 and HA-tagged Paxillin constructs were as described previously (15). To generate a pMXs-IP-based series of FLAG-tagged siRNA-resistant HIC-5 mutants (wild type, NLS, ΔLIM4, mLD3, and Cfl/ns), silent mutations were introduced into the siRNA target sequence (tct aac atc acg gac gaa atc, Hic-5; 262–278 nucleotides) at the underlined positions using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio, Inc., Otsu, Japan) with a mutated primer according to the manufacturer’s instructions.

**Real-time Reverse Transcription (RT)-PCR**—Total RNA was extracted from the cultured cells, reverse transcribed into cDNA, and analyzed by described previously methods (16).

**Antibodies and Immunoblotting**—The immunoblotting procedure was essentially the same as that described previously (15). Antibodies to p21<sup>Cip1</sup> (BD Biosciences, San Jose, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA), KLF4 (Santa Cruz Biotechnology, Santa Cruz, CA), HIC-5 (BD Biosciences), Lamin B1 (Invitrogen, Carlsbad, CA), and FLAG (Sigma) were used.

**Transfection and Infection**—siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The cells were transfected with siRNA duplexes (100 nM) and processed 48 h after transfection.

Viral infection was performed as described previously (17). In brief, Plat-E (for retrovirus) or HEK293T (for lentivirus) cells were transfected with viral vectors by the calcium phosphate method to obtain culture supernatants containing the virus. The target cells were infected by culturing for 24 h in virus-containing medium. Twenty-four hours after culture in the presence (Tet-Off lentivirus) or absence (Tet-On lentivirus, retrovirus) of Dox (1 µg/ml), the infected cells were selected and maintained in medium supplemented with 10 µg/ml blasticidin (lentivirus) or 5 µg/ml puromycin (retrovirus).

**Reporter Constructs and Assay**—The WWP reporter containing the 2.4-kbp upstream region of the p21<sup>Cip1</sup> promoter and the derived deletion reporters (WWP Sac, 2.0, 1.7, 1.4, Hinf, and Pst) were described previously (10, 18).

The pGL4.2/minP vector was generated by inserting the minimal promoter (aga ggg tat ata atg gca gtc cgg ctt cca g) immediately upstream of the luciferase reporter gene of pGL4.20 (Promega, Madison, WI). The WWP 2.0-Sac, 2.0–2.1-Sac, and 2.1-Sac reporters were generated by inserting the PCR-amplified fragments (−2243 to −1987 bp, −2243 to −2094 bp, and −2093 to −1987 bp, respectively) upstream of the p21<sup>Cip1</sup> promoter in the pGL4.2/minP vector. For a mutant series of the WWP 2.0-Sac reporter [KLF, USF, GATA, and Runt-related transcription factor 1 (RUNX1)] mt, point mutations were introduced into the wild-type WWP 2.0-Sac reporter as described above. The sequence information on the mutational changes is provided in Fig. 3A.

In the assay, firefly luciferase reporters (1 µg) together with the internal control of the Renilla luciferase reporter plasmid (pRL/CMV) (0.02 µg) were transiently introduced into the cells (19). Twenty-four hours after transfection, the cells were transferred to monolayer or suspension cultures and further incubated for 24 h. Luciferase activities were determined using the Dual Luciferase Assay Kit (Promega). Firefly luciferase activities were normalized with those of Renilla luciferase.

**5-Bromo-2’-deoxyuridine (BrdU) Incorporation**—BrdU incorporation was evaluated by immunocytochemistry as described previously (12).

**Chromatin Immunoprecipitation (ChIP) Assay**—A ChIP assay was performed based on the method described by Nelson et al. (20), with slight modifications (19). The antibody used for...
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this assay was anti-KLF4 (Santa Cruz Biotechnology), and antirabbit immunoglobulin (X0903; DAKO Japan, Kyoto, Japan) was used as the control.

Subcellular Fractionation—Subcellular fractionation was performed according to the method described by He et al. (21), with minor modifications. The cells were washed with ice-cold PBS and resuspended in CSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, and protease inhibitor mixture (Sigma)). After 5 min of incubation on ice, nuclear pellets were separated from cytoplasmic supernatants by centrifugation at 3000 × g for 10 min after washing with excess digestion buffer containing 250 mM ammonium sulfate. The chromatin fraction (soluble) was removed by centrifugation at 6500 × g for 10 min. The nuclear matrix was recovered by centrifugation at 6500 × g for 10 min after washing with excess digestion buffer containing 250 mM ammonium sulfate.

Statistical Analysis—Statistical differences were determined using the Student’s t test.

RESULTS

p21Cip1 Transactivation on Anchorage Loss Is Dependent on KLF4 and RUNX1, but Not p53, Binding Sites—It has been previously shown that p21Cip1 is up-regulated when anchorage-dependent cells are placed under suspension conditions (22). This leads to inhibition of CDKs such as CDK2 in a complex with cyclin E, which eventually results in growth arrest (23). In the present study, we confirmed p21Cip1 up-regulation at the mRNA and protein levels in C3H10T1/2 cells in response to anchorage deprivation (Fig. 1, A and B). Under the same conditions, the WWP reporter driven by the 2.4-kbp upstream region of p21Cip1 increased its activity, suggesting that induction was transcriptionally regulated (Fig. 1C). Similar transcriptional responses were observed in primary MEFs and TIG-7 cells (Fig. 1C). Another CKI, p27Kip1, was reportedly up-regulated by anchorage loss at the protein level (23), but not at the mRNA level (Fig. 1A). BrdU incorporation, which was ~70% in adherent cells, was lowered to less than 10% in nonadherent cells as reported previously (Fig. 1D). However, p21Cip1 siRNA treatment recovered incorporation to nearly 20% (Fig. 1D, Sus), suggesting that induced p21Cip1 contributed to growth arrest as described previously (24), although the extent was modest, possibly because of p27Kip1 compensation (22).

To elucidate the molecular mechanisms underlying the transcriptional response of p21Cip1 to anchorage loss, we performed the luciferase assay using a series of WWP deletion mutant reporters (Fig. 2A) and defined a regulatory region responsible for the response upstream of the gene. The results were definite as follows. The response of the WWP Sac reporter with the first 0.1 kbp (up to the SacI site at −2.2 kbp) truncated was comparable with that of full-length WWP, indicating that the first 0.1 kbp was dispensable for the response (Fig. 2B). In contrast, further deletion to −2.0 kbp (WWP 2.0) completely abolished the response, suggesting that the response was primarily regulated in the region between −2.2 (the SacI site) and −2.0 kbp. This region, designated as WWP 2.0-Sac, was found to independently exhibit a 2–4-fold response comparable with that of full-length WWP when connected directly to a minimal promoter (Fig. 2D), indicating that WWP 2.0-Sac was necessary and sufficient for the response. However, when split into two, neither 2.1–2.0-Sac nor 2.1–2.0-Sac could elicit a response (Fig. 2D), indicating that multiple (~two) sites located in either half cooperated to evoke the response. These reporter behaviors were basically recapitulated in primary MEFs (Fig. 2, B and D).

It should be noted that p53, albeit a well-established p21Cip1 transcription activator (25), was not likely involved in this response. Reporter assay results, for example, eliminated the necessity of two p53 binding sites. As shown in Fig. 2, A and B, the WWP Sac reporter with the first p53 site deleted retained nearly the complete response, whereas the WWP 2.0 reporter lost the response despite retention of the second site. In contrast, WWP 2.0-Sac, which contained no p53 binding sites, responded to detachment conditions comparably well with the intact WWP reporter as noted above (Fig. 2D). In addition, p53 siRNA treatment did not affect the p21Cip1 response (Supplemental Fig. S1), although the basal transcription level was reduced. These results consistently argued against p53 involvement in p21Cip1 transactivation on anchorage loss under the experimental conditions in the present study. Rather, the response was considered to be mediated by the WWP 2.0-Sac region, designated as a detachment-responsive (DR) region.

In further study focusing on the DR region, we searched for transcription factor binding sites (Fig. 3A) and introduced mutations in WWP 2.0-Sac reporter sequences as indicated in the inset (table). Among the mutations, those disrupting the KLF4 and RUNX1 sites caused distinctive effects. The KLF4 mutations almost completely deprived the reporter of the response (Fig. 3B, KLF4 mt1 and mt2), underscoring the importance of the sites for inducing the response. In contrast, the RUNX1 mutation led to a marked increase in the basal transcription level under Adh conditions (Fig. 3B, RUNX mt), indicating that a negative effect was imposed by this site under normal conditions. Importantly, no further increase in transcriptional activity occurred in this mutation on anchorage loss, suggesting that the RUNX1 site was simultaneously involved in response induction. Thus, the KLF4 and RUNX1 sites emerged as essential elements for the transcriptional response of p21Cip1 to anchorage loss. This result was consistent with the above observation, suggesting the requirement of multiple elements in the DR region for the response (Fig. 2, C and D). KLF4 involvement was also verified at the protein level because a decrease in the protein levels with shRNA expressed under the Tet-Off system (Supplemental Fig. S2A) resulted in marked deterioration of the p21Cip1 response (Fig. 3, C and D). With regard to RUNX1, we used pooled siRNA because of the unavailability of sequences for shRNA with a sufficient knockdown efficiency. Similar to the disruption of the RUNX1 site (Fig. 3B, RUNXmt), treatment with siRNA resulted in elevated basal levels of p21Cip1 expression (Fig. 3E), thus supporting the negative role of RUNX1.
HIC-5, a Molecular Scaffold for Transcriptional Complex Assembly, Is Required for the Transcriptional Response of p21Cip1—Our previous result implicated HIC-5 in the control of p21Cip1 transcription (10) and inferred its involvement in p21Cip1 transactivation on anchorage loss. In the present study, we examined this possibility and found that HIC-5 depletion by siRNA (supplemental Fig. S2B) significantly reduced p21Cip1 induction in nonadherent cells (Fig. 4A). Similarly, HIC-5 shRNA expression (Tet-On) (supplemental Fig. S2B) abolished a transcriptional response of the reporter driven by the DR region (WWP 2.0-Sac) (Fig. 4B, HIC-5; Dox+). These results underlined a pivotal role of HIC-5 in p21Cip1 transcriptional regulation in response to anchorage deprivation. The decrease of BrdU incorporation under anchorage-deprived conditions was alleviated by HIC-5 siRNA (Fig. 4C, Sus), which was expected from the inhibitory effect on p21Cip1 induction and was comparable with the effect of p21Cip1 siRNA (Fig. 1D, Sus).

Given the localization of HIC-5 at multiple cellular compartments and its shuttling ability (11), it was possible that HIC-5 regulated the transcriptional response either directly in the nucleus or indirectly by affecting cytoplasmic cellular events. To discriminate between these cases, we investigated whether
nuclear localization was a prerequisite for the HIC-5 function in p21<sup>Cip1</sup> induction. For this purpose, we prepared constructs expressing siRNA-resistant versions of wild- and mutant-types of HIC-5 (supplemental Fig. S2C) whose subcellular localizations were varied and examined their capability to substitute for endogenous HIC-5 depleted by siRNA. As expected, wild-type (WT) HIC-5 expression concomitant with siRNA counteracted the effect of siRNA and restored p21<sup>Cip1</sup> induction (Fig. 4D). The mLD3 and Cfl/ns mutants, both of which are defective in the nuclear export signal (NES) function and spontaneously accumulate in the nucleus (11), also showed this capability. Of note, the exclusively nuclear-localized version of HIC-5 (NLS) (10) similarly overcame siRNA effects, providing strong support for the concept that HIC-5 functions to directly promote transcription in the nucleus, but not at focal adhesions or by shuttling. Paxillin exhibited no such capability (Fig. 4D), thereby diminishing the concerns of artifacts because of protein overexpression. Among the HIC-5 mutants, ΔLIM4 was distinctive in that it lost the ability to substitute for endogenous HIC-5 despite retaining the nuclear localizing potential (11), suggesting the importance of this domain in the nuclear HIC-5 function. This is consistent with a previous result identifying
the LIM4 domain as essential for the scaffold function of HIC-5 in the transcriptional complex, although precise roles were unexplored, except for its role as an interface for protein oligomerization (15).

A Role of HIC-5 in Tethering KLF4 to the DNA Sites on Anchorage Loss—Because KLF4 and HIC-5 were found to play important roles in p21Cip1 up-regulation, we further investigated whether and how KLF4 and HIC-5 cooperated to achieve this function. Because the HIC-5 function targeted the transcriptional activity of the DR region (WWP 2.0-Sac) harboring the KLF4 sites (Fig. 4B), it was highly possible that HIC-5 regulated the behavior of the KLF4 transcription factor at a certain level. Considering the role of HIC-5 as a molecular scaffold for transcriptional machinery (10), HIC-5 may assist KLF4 recruit-
ment and/or retention in the p21Cip1 promoter region of the transcriptional complex. Alternatively, HIC-5 may regulate the KLF4 expression levels by modulating intracellular signaling, thereby affecting p21Cip1 expression indirectly.

To examine these possibilities, we first assessed the KLF4 expression levels with or without HIC-5 shRNA expression. As indicated in Fig. 5A, KLF4 expression was unaffected by the HIC-5 levels under these conditions, eliminating the possibility of HIC-5 regulation of the KLF4 expression levels. We next investigated the other possibility. We performed a ChIP assay and evaluated KLF4 binding to the p21Cip1 promoter region (Fig. 5B), in particular, the DR region, which contained two consensus sequences for KLF4 binding and was crucial for the response (Fig. 3, A and B). We also assessed binding to the...
proximal region encoding a cluster of six GC-rich sequences (Fig. 5B, Proximal) because the GC-rich sequences, originally identified as Sp1 binding sites (26), were recently shown to serve as KLF4 binding sites (27). The third target was set within the coding region as a negative control (Fig. 5B, CDS).

Results showed that KLF4 binding to the promoter region was remarkably stimulated by cellular detachment (Fig. 5B, Proximal) because the GC-rich sequences, originally identified as Sp1 binding sites (26), were recently shown to serve as KLF4 binding sites (27). The third target was set within the coding region as a negative control (Fig. 5B, CDS).

FIGURE 5. Role of HIC-5 in tethering KLF4 to DNA sites. A, TIG-7 cells infected with the lentivirus (constitutive) encoding HIC-5 shRNA (#1, #2) or the control (NC) were selected and pooled as described under "Experimental Procedures." After culturing under adherent (Adh) or nonadherent (Sus) conditions for 24 h, cell lysates were prepared and examined by immunoblotting with the indicated antibodies. GAPDH is shown as a loading control. B, the detachment-responsive element (DRE) (see text) and Sp1 binding sites in the p21Cip1 promoter and the primers (arrows) used in the ChIP assay are illustrated. C, the pooled cells in A were cultured under Adh and Sus conditions for 24 h and lysed after fixation with 1% formaldehyde. The lysates were subjected to ChIP assay as described under "Experimental Procedures." The lysates were prepared and examined by immunoblotting with the indicated antibodies. Lamin B1 and GAPDH serve as fractionation monitors as well as loading controls. E, C3H10T1/2 cells were infected with the retroviral vectors for the FLAG-tagged wild-type or the LIM4-deleted mutant (ΔLIM4) HIC-5 as described under "Experimental Procedures." After selection, the pooled cells were incubated, fractionated, and analyzed as in D.

Results showed that KLF4 binding to the promoter region was remarkably stimulated by cellular detachment (Fig. 5C, NC; Adh versus Sus). Of note, the response to detachment was completely abolished by HIC-5 shRNA expression, suggesting that HIC-5 played an important role in facilitating KLF4 binding to DNA in nonadherent cells. Binding was increased almost equally in both DR and proximal regions. Binding enhancement in the proximal region was apparently incompatible with the exclusive dependence of the transcriptional response on the DR region, but not on the proximal region (Fig. 2, A and B). This inconsistency can be explained by the differences in surrounding sequences of the two regions. The DR region, but not the proximal region, contained the RUNX1 site, another essential element collaborating with the KLF4 sites for the response (Figs. 2, C and D and 3B) in the vicinity of the KLF4 sites. Alternatively, the KLF4 sites in the two regions could be redundant. However, the mutation at the KLF4 site in the DR region sig-
significantly impaired the WWP full-length reporter response (supplemental Fig. S2E), making the possibility unlikely.

Next, we investigated the mechanism underlying the HIC-5 function of tethering KLF4 to the sites. A clue was obtained when examining cellular HIC-5 localization through biochemical fractionation. In adherent cells, HIC-5 was mostly present in the cytoplasm, as expected from its primary localization at focal adhesions (Fig. 5D, Adh). Intriguingly, in nonadherent cells, a significant population changed its localization to the nucleus, specifically to the matrix fraction (Fig. 5D, Sus). In case of the functionally defective ΔLIM4 mutant (Fig. 4D), however, the protein largely remained in the cytoplasm even under Sus conditions with a little increase in the nuclear matrix (Fig. 5E), indicating that the nuclear matrix localization of HIC-5 in response to detachment was mediated by LIM4. More importantly, it suggests that the HIC-5 function in the nuclei of nonadherent cells is based on its nuclear matrix localization.

**DISCUSSION**

**DR Element (DRE) Identified in the p21<sup>Cip1</sup> Promoter Region**—A line of evidence has shown that cellular detachment from ECM activates transcriptional responses, some of which are engaged in subsequent growth arrest or cell death (29, 30). However, detailed mechanisms underlying the responses remain largely unknown. In the present study, we examined p21<sup>Cip1</sup> transcriptional up-regulation in cells deprived of ECM attachment and first identified the upstream regulatory region (designated as DR) that mediated the transcriptional response to disruption of cell-ECM interactions. The DR region contains the KLF4 and RUNX1 sites in close proximity, both of which are necessary for the response but are individually insufficient (Fig. 2, C and D) and believed to constitute a “DRE” together. A similar disposition of the RUNX1 and KLF4 sites is found downstream of the p53 binding site in murine p21<sup>Cip1</sup> genomes (Fig. 7B), suggesting the evolutionary conservation of DRE across the species. It is also interesting to note that among the tested transcriptional targets of KLF4 (31), half (4/8) responded to loss of anchorage (data not shown).

In our study, p53 involvement was consistently unlikely (supplemental Fig. S1 and Fig. 2) and seemingly contradictory to a previous report (22) in which the authors reached their conclusion largely on the basis of p53-null cell responses. Given the requirement of p53 for the basal transcriptional activity of p21<sup>Cip1</sup> (32, 33) (supplemental Fig. S1), complete elimination of p53 possibly deprived cells of the fundamental transcriptional potential undermining response induction. Another concern was the possibility that the KLF4 and RUNX1 sites immediately adjacent to the p53 site were deleted together with the p53 site in their mutant reporter that lost the response. A lack of detailed information on the constructs hampered further discussion.

**A DR Transcriptional Mechanism Directed by a Molecular Scaffold, HIC-5**—The most prominent finding of the present study was a novel mechanism regulating transcription in a detachment-dependent manner with the aid of a molecular scaffold, HIC-5. HIC-5 assists in recruitment and/or retention of transcription factors at a given DNA site in response to cellular detachment. In mechanistic detail, the pivot is the LIM4 domain, a mutant of which lost the potential to stimulate p21<sup>Cip1</sup> transcription concomitantly with the loss of oligomerization abilities and nuclear matrix localization (10) (Figs. 4D and 5E). Likewise, paxillin, despite high homology to HIC-5, did not stimulate p21<sup>Cip1</sup> transcription (10) and exhibited neither oligomerization (15) nor nuclear matrix localization (data not shown). This fact provides another instance correlating the HIC-5 function as a scaffold with inherent oligomerization and nuclear matrix localization properties, and along with the case of the LIM4 mutant, strongly supports the hypothesis that HIC-5 oligomerizes and consequently localizes to the nuclear matrix, thereby achieving its function. Accordingly, the primary force driving the system is hypothetically HIC-5 oligomerization in response to anchorage loss. In general, protein oligomerization is promoted by an increase in protein concentration. In case of HIC-5, anchorage loss triggers spontaneous nuclear accumulation of the protein, i.e. an increase in concentration in the nucleus, because of NES inactivation (12). Such conditions expectedly accelerate oligomerization and consequently, nuclear matrix localization of HIC-5, which in turn, presumably stabilizes its scaffold architecture and the transcriptional complex including KLF4 on DNA. Because HIC-5 and KLF4 did not appear to make direct contact (data not shown), the transcriptional complex is believed to include additional factors such as nuclear matrix components. Clarification of this transcriptional complex entity is necessary to elucidate this mechanism completely.

In addition, the present study revealed a novel aspect of p21<sup>Cip1</sup> transcriptional regulation, which operated at the RUNX1 site in the DR region to repress basal transcription under Adh conditions (Fig. 3B). CRP2 was identified as a candidate repression regulator (Fig. 6, A and B), although its precise role remains unclear. Because p21<sup>Cip1</sup> basal level transcription was unaffected by HIC-5 knockdown (Fig. 4, A and B) and similarly by KLF4 knockdown (Fig. 3, C and D) and mutation at
the KLF4 sites (Fig. 3B), neither HIC-5 nor KLF4 were likely related to negative regulation at the RUNX1 site under Adh conditions. Rather, in response to detachment, HIC-5 and KLF4 are recruited to the KLF4 sites and may function to eliminate the negative effect on the adjacent RUNX1 site, thereby stimulating DRE transcriptional activity under the conditions (Fig. 7A).

In conclusion, HIC-5 has emerged as a novel molecular adapter directly coupling the cellular adhesion status to transcription and eventually contributing to anchorage dependence of cell growth. Together with the previous study (12), a molecular function of HIC-5 could be summarized as a bimodal organizer of a fail-safe system simultaneously targeting two crucial cell-cycle regulators, cyclin D1 and p21Cip1. Such a protective system is pivotal for homeostasis of multicellular organisms. Even under physiological conditions, for instance, when cells move toward a chemoattractant or enter the mitotic phase, cells undergo regional or global provisional loss of ECM contact. Under pathophysiological circumstances such as postinflammatory scarring, cells are inevitably exposed to dynamic changes in surrounding ECM compositions. Therefore, an increased understanding of cellular behaviors upon interference with proper ECM attachment and of underlying mechanisms is not only important in a biological sense but also essen-

![Graph A](image-url-a)

**FIGURE 6.** Negative regulation at the RUNX1 site by the LIM-only protein CRP2. A, primary MEFs were infected with the lentivirus (Tet-Off) encoding shRNA against CRP2 (#1, #2) or EGFP (a control). After selection, the cells were pooled and cultured for 48 h in the presence or absence of 1 μg/ml Dox as above. The p21Cip1 mRNA levels were quantified by real-time RT-PCR. The values (mean ± S.D.) are relative to the control (EGFP, Dox +). B, the cells obtained in A were transiently transfected with the wild-type (WT) or the RUNX1 mutant (mtRUNX1) WWP 2.0-Sac reporter plasmid (Fig. 3A) together with the internal control in the presence or absence of 1 μg/ml Dox. After 24 h, reporter activities were measured and graphed as above. The values (mean ± S.D.) are relative to the control (shEGFP, WT; Dox -) (#, p < 0.05). The status of CRP2 expression under Dox+ conditions is shown below the graph. C, the cells obtained in A were transiently transfected with WT or the KLF4 mt2 mutant (mtKLF4) WWP 2.0-Sac reporter plasmid (Fig. 3A), and reporter activities were examined as in B (#, p < 0.05). Each assay was performed in triplicate and repeated at least three times.
tial for therapeutic development for these pathological conditions. HIC-5 could be a key modulator of cellular behavior under such pathophysiological circumstances.

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