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14. ABSTRACT
Increased genomic instability arising from centrosomal amplification has been proposed to be an important factor causing development of traits associated with highly malignant ovarian tumors, including multidrug resistance and increased tendency to metastasis. This proposal addresses the hypothesized interaction between the Cas proteins (HEF1 and p130Cas), Aurora A (AurA) and Ajuba as being likely to contribute to genomic instability and metastatic properties of ovarian tumors. Our work in the second project period has defined and performed detailed analysis of the HEF1-AurA-Ajuba complex. This has allowed us to develop a model in which HEF1 and AurA mutually stabilize each other, and HEF1 and Ajuba synergize to promote AurA activation at mitotic entry. At mitotic exit, phosphorylation by AurA promotes HEF1 return to focal adhesions. Excess of HEF1 protects AurA from inhibition by targeted small molecule inhibitors. In the past two years, elevation of HEF1 has been established as important for metastasis and/or invasion in lung cancer, melanoma, and glioblastoma; our ongoing work on this project will determine whether this is also true for ovarian cancer.

15. SUBJECT TERMS
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INTRODUCTION: The goal of this proposal is to better understand factors leading to ovarian cancer development, with the intent of improving diagnosis and treatment for ovarian cancer. Our preliminary data defined the HEF1 member of the Cas protein family as a regulator of centrosomal dynamics and genomic instability through control of the Aurora A (AurA) kinase. The goal of the proposal was to explore HEF1 and p130Cas protein status as a contributing factor to early onset of ovarian cancers, and use this information to assess the value of combining targeted small molecule therapeutics for ovarian cancer therapy. Three Aims were proposed to address this objective. In *aim 1*, we proposed to examine tumor samples to determine if HEF1 and/or p130Cas expression, AurA activation, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis. In *aim 2*, we proposed to examine the mechanism by which Cas proteins activate AurA, and determine if drug-mediated inactivation of AurA inhibits Cas promotion of aneuploidy. In *Aim 3*, we proposed to use further drug and depletion experiments to determine if centrosome amplification and enhanced cellular metastasis are linked, and dependent on Cas/integrin signaling, or whether these are separable properties; and to evaluate combination of AurA- and integrin- directed therapies.

BODY: During the most recent reporting period (11/06-11/07), we have almost completed work on Aims 2 and 3, resulting in a research publication in Cell and reviews. An additional research publication is in preparation. Because of our focus on these two aims, we did not extensively advance our work on Aim 1. We are requesting a *one-year, no-cost extension* of this grant to allow us to complete this work.

Aim 1: Determination of whether HEF1 or p130Cas expression, activated AurA, and centrosomal amplification are linked, and whether Cas protein upregulation is associated with a poor prognosis. Limited progress this year: more in 2008.

Aim 2: Determining the mechanism by which HEF1 and p130Cas proteins activate AurA, and whether inactivation of AurA inhibits Cas promotion of aneuploidy. This Aim had several sub-Aims: 1. Do HEF1 and AurA directly associate? 2. Does p130Cas associate with AurA? 3. Does addition of HEF1 or p130Cas to AurA induce the kinase activity of AurA in vitro? 4. Is HEF1 itself is an AurA substrate? 5. Does HEF1 associate with Ajuba? 6. Does inhibition of the AurA kinase inhibit the centrosomal amplification and aneuploidy induced by HEF1 overexpression?

As of the last progress report, we had predominantly addressed sub-Aims 1-5, with some of this work in initial publications [1, 2] HEF1 and AurA interact, with HEF1 required for AurA activation; p130Cas does not interact with AurA. HEF1 is an AurA substrate; phosphorylation of HEF1 by AurA causes the two proteins to bind each other with lower affinity. The biological significance of these findings is discussed at length in the provided manuscripts. In the past year, we have continued our work on points 5 and 6, studying the interdependence of HEF1, Aurora A, and Ajuba; this Aim is now complete. Key results from our manuscript in preparation are stated below: we are currently assembling final, publication-quality figures.

Ajuba and HEF1 directly interact, and synergistically activate AurA. The Ajuba scaffolding protein has the classical structure of LIM-family proteins, marked by an extended gly/pro-rich N-terminus, and three tandem LIM protein interaction domains at the C-terminus[3]. HEF1 is also an adaptor protein, encompassing an N-terminal SH3 domain, an extended "substrate domain" containing many binding motifs for proteins with SH2 domains, followed by additional protein interaction sequences[4, 5]. To begin to evaluate HEF1 interactions with Ajuba, we first used HEF1- and Ajuba-directed antibodies to co-immunoprecipitate endogenous Ajuba and HEF1 from MCF7 cells. In each case, we observed dose-dependent co-immunoprecipitation of the two proteins. The observed interaction might be direct, or might instead be mediated through the common interactor, Aurora A (AurA). We next used an *in vitro* binding assay with purified GST-fused derivatives of HEF1 and FLAG-tagged derivatives of Ajuba (Aj). FLAG-Ajuba and its derivatives pulled down amino-terminal fragments of HEF1 ($HEF1_{1-363}$ and $HEF1_{82-398}$) and $HEF1_{351-653}$, implying direct interaction potential.

Both HEF1[1] and Ajuba[6] independently activate AurA kinase in *in vitro* assays. We compared the ability of Ajuba and HEF1 alone and in combination to activate AurA, using the same fragments of each protein previously described as activating in vitro. These HEF1 and Ajuba fragments strongly synergized in activating AurA, raising the possibility that HEF1 and Ajuba might cooperate to activate AurA in cell cycle.

An Ajuba-HEF1-AurA complex is abundant at the G2/M boundary. The peak of AurA activation by HEF1 and Ajuba occurs at G2/M boundary, paralleling maximal accumulation of HEF1 in cycling cells[7]. Using cell lysates synchronized either in G1 by thymidine treatment, or at the G2/M boundary by nocodozole treatment, we found significantly greater levels of association between HEF1, Ajuba, and AurA in G2/M-synchronized cells, than in G1 cells.

To determine whether the co-immunoprecipitation-based interactions represented the simultaneous co-existence of HEF1, Ajuba, and AurA in a ternary complex, or instead represented multiple binary interactions among these proteins, we employed 2D gel analysis. MCF7 cells were synchronized by double thymidine block, released, and samples collected at intervals corresponding to G1/S and G2 phase, as confirmed by parallel FACS analysis. Contrast of the G1/S and G2/M gels indicated that HEF1, Ajuba, and AurA were all constituents of a complex that migrated at ~ 160 kDa on a native gel. Of the two documented HEF1 forms, p115 and p105, only the higher molecular weight form, which has previously been shown to be most abundant in G2 [7], was detected in the complex. The 160 kDa complex was detected in both G1/S and G2/M phase lysates, but was much more abundant in G2/M lysates.

In G1/S cells, p115 HEF1 was also found in at least 2 additional anomalously high molecular weight complexes, migrating at ~ 200 kDa and 180 kDa, respectively. Neither of these complexes contained Ajuba or AurA; most likely, these complexes reflect HEF1 associations with partners including FAK, Src, and Crk, in the context of HEF1 interphase functions at focal adhesions (analogous to the situation for p130Cas, see [8]). Quantification of the HEF1 signal indicated that only 25 % of HEF1 signal was associated with the Ajuba/AurA complex, while almost all Ajuba and AurA were in the 160 kDa complex. All the p105 HEF1 detected migrated as a monomeric species, suggesting it was not involved in any stable protein interactions. By contrast, in the late

G2 cell lysates, almost no p105 HEF1 was detected, and the higher molecular weight complexes were absent. Rather, this analysis resolved two closely migrating HEF1 species, p115 and a novel, ~p113 kDa: all the p115 HEF1 was concentrated in the 160-kDa complex containing AurA and Ajuba.

A complex between AurA and HEF1 is important for mutual stabilization of the proteins in vivo. HEF1 and Ajuba binding may increase AurA activity at mitotic entry in vivo by recruiting additional activating proteins, by directly inducing a conformational change in AurA conducive to activation, by increasing the level of total AurA, or by a combination of these effects. To dissect the mechanism of HEF1 action, we used siRNA to deplete HEF1, Ajuba, and AurA in cells pre-synchronized by arrest in thymidine, and examined total levels of each protein. We found that depletion of HEF1, but not Ajuba, reduced levels of AurA; and that depletion of AurA, but not Ajuba, reduced levels of HEF1. Treatment of cells with the proteasomal inhibitor ALLN reversed this effect. siRNA depletion of HEF1 and AurA did not reciprocally reduce HEF1 and AurA mRNA expression. Finally, FACS analysis confirmed all cells were in a comparable phase of cell cycle, excluding potential indirect effects due to the cell cycle-dependent accumulation of HEF1 and AurA. Together, these data indicated that HEF1 and AurA mutually stabilized each other from protein degradation.

We then asked if HEF1 or Ajuba overexpression increased cellular levels of AurA, or whether AurA overexpression increased HEF1 levels. HEF1 overexpression significantly increased endogenous levels of AurA, while Ajuba overexpression did not. This HEF1-dependent increase required the ability of HEF1 to bind AurA, as overexpression of a S296E HEF1 derivative, previously shown to reduce HEF1-AurA interactions REF, did not increase AurA expression. Finally, for both HEF1 overexpressing and depleted cells, we assessed the ratio of total to T^{288}-phosphorylated (active) AurA. These data indicated the overall ratio of active to inactive AurA was also reduced. This analysis suggested in vivo, HEF1 both stabilized and activated AurA.

Cells overexpressing HEF1 have elevated AurA expression. We next treated HEF1-overexpressing cells with small molecule inhibitor of AurA. This compound, PHA 680632 did not affect the levels of HEF1 associated with AurA in G2 cells, nor the overall levels of HEF1 in the cell. Interestingly, PHA680632 was not able to decrease activity of AurA in HEF1-overexpressing cells, at concentrations at which it fully inhibited AurA in control cells. These data imply that HEF1 stabilizes the active conformation of AurA.

In summary, our results to date strongly validate the idea of a HEF1-Ajuba-AurA machine that times mitotic entry. They indicate that the abilities of HEF1 and AurA to induce genomic instability and cancer are likely to be linked, and strongly suggest that Ajuba is a critical component of the interaction network, that may be targeted in HEF1-AurA-associated cancers. They also suggest that there may be difficulties in using small molecule inhibition of AurA in cancers involving HEF1 overexpression.

<u>Aim 3: Determination of whether if centrosome amplification and enhanced cellular metastasis are linked, and dependent on HEF1/integrin signaling, and whether combination of integrin-pathways targeted inhibitors with AurA inhibitors is of clinical</u>

merit. This Aim had the following sub-Aims: 1. We will perform structure-function analysis to evaluate whether HEF1 control of cell attachment, and regulation of centrosome-associated functions utilize overlapping or separable domains. 2. We will determine if inhibition of HEF1 signaling by inhibitors targeting the integrin pathway blocks HEF1 ability to induce centrosomal amplification. 3. We will determine whether inhibition of AurA signaling blocks HEF1-dependent cell migration and invasion. 4. We will determine whether combination of AurA- and integrin-targeted inhibitors synergistically blocks both cell migration/invasion, and centrosomal amplification.

As of the last progress report, we had completely addressed sub-Aim 1, with this data included in the publication supplied: different domains of HEF1 are required for the two functions [1]. We have next systematically analyzed the role of AurA in HEF1-dependent cell attachment. While we need to complete work on sub-Aims 2 and 4, and will in the next year, we have concluded that AurA phosphorylation of HEF1 contributes to HEF1 return to focal adhesions at the end of mitosis, but not HEF1 activity later in the cell cycle, such as promotion of migration. Results from our manuscript in preparation:

Phosphorylation of HEF1 by AurA allows post-mitotic re-localization of HEF1 at sites of focal adhesion. We previously showed that AurA phosphorylation of HEF1 on S^{296} reduced HEF1-AurA interaction affinity, and a HEF1 S296E phosphomimic derivative did not interact well with AurA. Mitotic phosphorylation of HEF1 on S296 may modify HEF1 activity during or after mitosis, including regulating integrity of the Ajuba-AurA-HEF1 complex. To address these questions, we developed a phospho-specific antibody against S^{296}-HEF1. This antibody, PS296, specifically recognized endogenous HEF1 in mitotic but not G1 phase cell lysates. PS296 also recognized overexpressed GFP-HEF1 and GFP-HEF1(S296E), but not GFP-HEF1(S296A) overexpressed in HEK293 cells; recognition of GFP-HEF1 was lost in cells pre-treated with AurA kinase inhibitor.

We then used PS296 to examine the localization of S^{296}-HEF1 in MCF7 cells synchronized at different stages of cell cycle. S^{296}-HEF1 was abundant in mitotic cells at the mitotic spindle and midzone, comparable to total HEF1. Strikingly, S^{296}-HEF1 concentrated at the "tips" of re-forming focal adhesions in post-mitotic cells in early G1, although little or no S^{296}-HEF1 was seen at focal adhesions in asynchronously growing or thymidine blocked cells. These results suggested that HEF1 phosphorylation by AurA could be an important step for translocation of HEF1 at the end of mitosis to newly formed focal adhesions, by providing an additional localization cue. To support this interpretation, we examined the intracellular localization of over-expressed GFP-tagged HEF1, HEF1(S296A), and HEF1(S296E). Both mutants and wild type HEF1 localized to the centrosome, comparably with GFP-HEF1, demonstrating the AurA phosphorylation site did not promiscuously influence localization of these proteins and in agreement with the earlier observation that HEF1 accumulates at the centrosome before AurA becomes active in the cell cycle in late G2. HEF1(S296A) was more diffusely localized in the cytoplasm relative to wild type HEF1 and failed to concentrate as effectively at focal adhesions as HEF1, while the opposite was seen with HEF1(S296E). This implied that phosphorylation of S296 provided a separate cue for focal adhesion association. Finally, treatment of interphase cells with PHA680632 had no observed effect on cell migration.

Like HEF1, Ajuba functions at focal adhesions and in cell migration, raising the possibility that HEF1 and Ajuba may co-localize to focal adhesions following mitosis. Antibodies to Ajuba had high background for immunofluorescence in post-mitotic cells, limiting ability to directly assess this point. However, based on co-immunoprecipitation, neither an S296E nor an S296A mutation inhibited the ability of Myc-Ajuba to associate with GFP-HEF1. In complementary experiments, we compared the coimmunoprecipitation of HEF1 and Ajuba in reactions containing AurA and ATP (i.e., catalytically active AurA), and the presence of a catalytically inactive mutant of AurA, versus wild type AurA. Finally, overexpressed Ajuba and HEF1 co-localized in vivo. All of these experiments suggested that Ajuba continued to interact with HEF1 in establishment of focal adhesions in post-mitotic cells.

In summary, these data continue to support the idea that HEF1 interactions with AurA in mitotic control are separated from HEF1 interactions with FAK and Src in cell migration, while HEF1 interactions with Ajuba may be relevant in each context. A particularly interesting observation is that AurA controls HEF1 return to focal adhesions at the end of mitosis: defects in the traction forces generated at focal adhesions has previously been described as one major source of cytokinetic failure. We believe that deregulation of this process in HEF1 and/or AurA overexpressing cells may complement defects in spindle integrity in contributing to genomic instability. Finally, our ongoing work on the association of HEF1 and AurA has defined a completely unexpected role of HEF1 and AurA in regulation of cell cilia. These findings, discussed below in the **CONCLUSION** section, have provided an important new dimension to our thinking about HEF1 and AurA in cancer.

KEY RESEARCH ACCOMPLISHMENTS:

First funding period

- We have optimized conditions for screening tumor lysates by Western blot for expression of all the proteins noted in the Aims.
- We have identified a statistically significant negative correlation between HEF1 overexpression and Aurora A overexpression.
- We have begun to analyze HEF1/AuroraA/centrosome expression in tissue microarrays.
- We have demonstrated a critical interaction between HEF1 and Aurora A that is necessary for Aurora A activation and mitotic progression.
- We have demonstrated that HEF1 interacts with Ajuba, with HEF1 and Ajuba synergizing to activate Aurora A.
- We have mapped the domains of HEF1 required for action at centrosome and focal adhesions, and we have shown that these are separable.

Second Funding period.

- We have fully mapped the domains for interactions between HEF1, AurA, and

Ajuba.

- We have used 2D gel analysis to demonstrate that HEF1 interacts endogenously with AurA and Ajuba in a complex that predominates in G2 phase cells, but is also detected earlier in cell cycle.
- We have demonstrated mutual interdependence of HEF1 and AurA for protein stability, and for AurA activation.
- We have determined that AurA phosphorylation of HEF1 is important for HEF1 relocalization to focal adhesions at cytokinesis, and may contribute to the traction forces allowing resolution of cell separation.
- Current data do not support the idea that AurA contributes in a meaningful way to HEF1-dependent cell migration.
- As summarized below (CONCLUSIONS), we have defined a completely novel role for HEF1-AurA interactions in regulation of cell cilia.

REPORTABLE OUTCOMES:

The first funding period of this project resulted in two manuscripts published in Nature Cell Biology and in Cell Cycle[1, 2]. In the current funding period, we have published results arising from our work for Aims 2 and 3 in Cell[9], and additionally have published 2 reviews[5, 10], with a third accepted pending minor revisions[11]. An additional manuscript, exploring the HEF1-Aurora A-Ajuba interactions in greater depth, is in preparation, with an intended submission date in December 2007. In 2007, data from this project has been presented in invited talks at the FASEB Conference on Cilia and Flagella (Saxtons River, Vermont), and at the Lankenau Institute (Philadelphia), Louisiana State University, Moffitt Cancer Center (Florida), University of Pennsylvania, University of Queensland (Brisbane, AU), and in other places. The work will also be presented in 2 posters at the December 2007 annual meeting of the American Society for Cell Biology. Data from this project has been used to support an RO1 grant application to the NIH for extension of our results in the area of polycystic kidney disease, and in support of the renewal of an existing RO1.

CONCLUSION:

Our study has validated a completely novel mechanism of activating AurA at the centrosome, through use of HEF1 and Ajuba. We have also defined a new role for AurA in promoting cellular attachment by allowing HEF1 to associate with focal adhesions These extremely important findings connect cell adhesion and cell cycle signaling, and provides insight to the linked deregulation of these processes in cancer. We have expanded on these ideas in a number of recent reviews[5, 10-12].

For further context, we would note two additional points describing work beyond the immediate scope of the proposal. First, over the past two years, HEF1 (also known as NEDD9) has been identified as a major pro-metastatic factor for melanoma[13]. We have collaborated with the Gladson laboratory to show that HEF1 contributes in an important way to the invasive behavior of glioblastomas[14]. HEF1 overexpression marks lung cancer associated with mutation of the LKB1 tumor suppressor[15]. Our own work, beyond the scope of this proposal, has established using a mouse model that HEF1 null status

dramatically reduces the rate of tumor growth for breast tumors in the MMTV-PyVT cancer model; we will be preparing a manuscript describing these exciting results in approximately 4 months. All of these data support the idea that HEF1 is an important factor in the progression and metastasis of many cancers. Based on the data summarized above, and this compelling evidence for the importance of HEF1 in metastasis, we have initiated a collaboration with Denise Connolly to cross HEF1-/- mice to her MISIIR model for ovarian cancer[16]; we hope to begin this work, which will reinforce the goals of this proposal, in the next few months.

Second, building from our identification of HEF1 as an activator of Aurora, we have now defined a HEF1-Aurora A signaling switch as a major determinant of ciliary disassembly. Immotile primary cilia are found on most epithelial and stromal cells in the mammalian body[17]. The primary cilium is a centriole-based organelle that consists of (9+0) microtubule pairs located at the plasma membrane. The past decade has seen exponential growth in studies of the cilium, based on the recognition that numerous developmental defects and chronic diseases arise from aberrant formation or function of cilium[18, 19]. The idea of the cilium as 'cellular cybernetic probe' capable of transducing environmental information from the extracellular matrix to the centrosome, and suggesting a matrix-cilium-Golgi signaling continuum[20], first proposed in the 1990s, is now supported by extensive data, and informs studies of “ciliary diseases” such as PKD, Bardet-Biedl syndrome, and Kartagener’s syndrome.

A correlation between cellular transformation and loss of a primary cilium has been noted for over a decade[21]. It has been proposed that cancer cells lacking cilia reduce or alter their response to extracellular cues that regulate growth and differentiation. A number of recent studies have shown that a number of critical cell signaling and adhesion molecules are clustered on the cilium, and require an intact cilium for normal function (Figure 1A). These include components of the Shh (Sonic Hedgehog), PCP (planar cell polarity), PDGFRα (platelet derived growth factor receptor α) and VHL/GSK3β (von Hippel-Lindau, and glycogen synthase kinase 3 β) pathways[22-24]. For at least VHL/GSK3β, deregulated expression of the signaling protein has been found to influence the ability of cells to form cilia [25]. Nevertheless, the significance of ciliary loss for influencing signaling in cancer development has been largely unclear.

In the past year, we defined[9] a novel a HEF1 – Aurora A – HDAC6 signaling axis governing the resorption of cilia, which suggests a novel molecular mechanism to explain at least some cancer-associated ciliary loss. We have proposed that enhanced intracellular levels of HEF1 and Aurora A interfere with a normal cellular cilia-centriole-centrosome oscillation that helps time cell cycle by staging critical signaling complexes that govern emergence from quiescence, entry to S phase, and initiation of M phase. Through these actions, HEF1 and Aurora A can provide an additional stimulus to cancer aggressiveness, beyond promotion of cell invasion and induction of genomic instability. The conclusion of the present studies will help provide the justification for combined use of clinical agents targeting Aurora A, integrins, and potentially histone deacetylases as tools to combat cancer.

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Cell

HEF1-Dependent Aurora A Activation Induces Disassembly of the Primary Cilium

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SUMMARY

The mammalian cilium protrudes from the apical/lumenal surface of polarized cells and acts as a sensor of environmental cues. Numerous developmental disorders and pathological conditions have been shown to arise from defects in cilia-associated signaling proteins. Despite mounting evidence that cilia are essential sites for coordination of cell signaling, little is known about the cellular mechanisms controlling their formation and disassembly. Here, we show that interactions between the prometastatic scaffolding protein HEF1/Cas-L/NEDD9 and the oncogenic Aurora A (AurA) kinase at the basal body of cilia causes phosphorylation and activation of HDAC6, a tubulin deacetylase, promoting ciliary disassembly. We show that this pathway is both necessary and sufficient for ciliary resorption and that it constitutes an unexpected nonmitotic activity of AurA in vertebrates. Moreover, we demonstrate that small molecule inhibitors of AurA and HDAC6 selectively stabilize cilia from regulated resorption cues, suggesting a novel mode of action for these clinical agents.

INTRODUCTION

In polycystic kidney disease (PKD), Bardet-Biedl Syndrome (BBS), and other disorders, mutations in cilia-associated structural or signaling proteins cause insensitivity to external mechanical and diffusible signaling cues, resulting in disorganized, hyperplastic cell growth (Benzing and Walz, 2006; Pan et al., 2005; Singla and Reiter, 2006). On the organismal level, ciliary defects produce renal cysts, infertility, respiratory disorders, *situs inversus*, and predisposition to obesity, diabetes, and hypertension. Notably, recent studies have shown that the Hedgehog, Wnt, PDGF$\alpha\alpha$, and other signaling cascades are coordinated at cilia (Cano et al., 2004; Huangfu and Anderson, 2005; Liu et al., 2005; Schneider et al., 2005; Simons et al., 2005; Tanaka et al., 2005). The frequent deregulation of these pathways during cell transformation, together with the common disappearance of cilia in transformed cells, raises the possibility that defective ciliary signaling may promote cancer.

Although an increasing number of proteins are being defined as ciliary structural components or cilia-associated signaling proteins, very little is currently known about the cellular machinery controlling the formation and resorption of cilia. It has long been known that cilia are regulated dynamically throughout the cell cycle. In many cells, resorption occurs at mitotic entry, and reappearance after progression into G1. However, resorption is not solely linked to mitotic entry, with some cells undergoing waves of resorption at different points in cell cycle: for example, Tucker et al. have noted ciliary resorption as cells emerge from quiescence, prior to S-phase (Quarmby and Parker, 2005; Rieder et al., 1979; Tucker et al., 1979). Given their increasingly apparent role in detecting and transmitting extracellular signals, regulated formation, disassembly, or shortening of cilia may play an important role in cellular growth controls, serving as a rheostat to limit response to overly persistent or abnormal cell growth cues in the extracellular environment.

A cilium arises from a basal body, a structure that differentiates from one of the centrioles in the centrosome in nonproliferating cells and organizes the microtubule bundles that constitute the ciliary axoneme. Cilia are evolutionarily related to the motile flagella of lower eukaryotes, such as the green algae *Chlamydomonas.* Genetic studies in *Chlamydomonas* have recently begun to dissect the process of flagellar resorption (Bradley and Quarmby, 2005; Marshall et al., 2005; Pan and Snell, 2005; Quarmby, 2004). These studies have identified altered functionality of the intraflagellar transport (IFT) machinery and destabilization of the axoneme as hallmarks of disassembly, and implicated CALK and other kinases as regulators of disassembly. The means by which CALK becomes activated at initiation of disassembly and the critical CALK effectors in the disassembly process remain unknown, as does the relevance of these observations to higher eukaryotes.

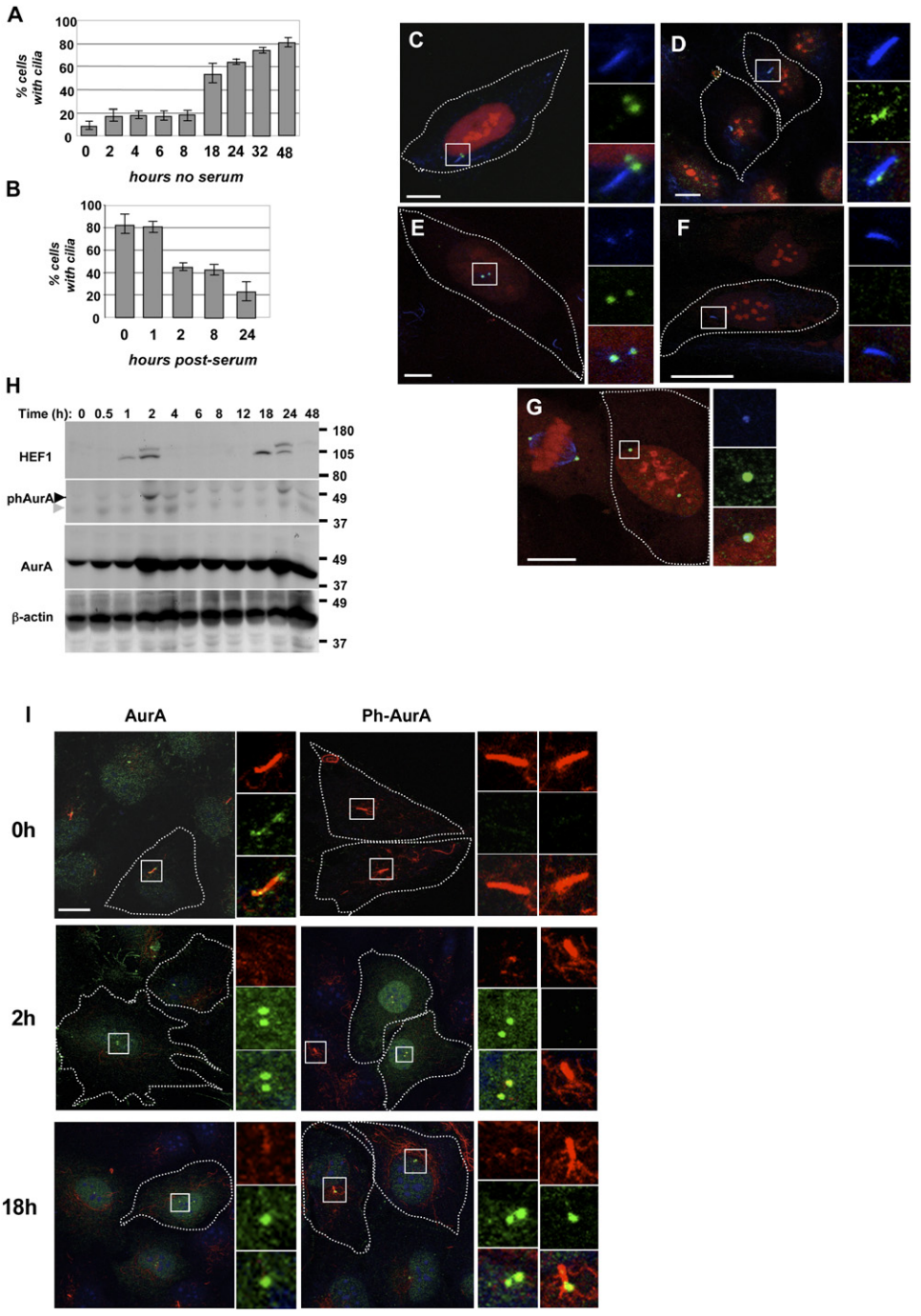

Figure 1. Activation of AurA at the Basal Body Occurs during Ciliary Disassembly

(A) Assembly of cilia. An average of 200 cells were counted in two independent experiments, error bars show the SD.

(B) Disassembly of cilia induced by serum stimulation. An average of 150 cells were counted in each of four experiments, error bars show the SD.

(C) Immunofluorescence of quiescent cells with antibody to AurA (green), acetylated α-tubulin (blue), and DNA (red). The scale bar represents 10 μm. In this and subsequent panels, boxes in main image indicate structures shown at high magnification to right.

(D) Immunofluorescence of quiescent cells with polyclonal rabbit antibody to HEF1 (green), also visualizing acetylated α-tubulin (blue), and DNA (red); compare also to (E). The scale bar represents10 μm.

(E) Immunofluorescence of quiescent cells with monoclonal antibody to HEF1 (green), also visualizing γ-tubulin (blue) and DNA (red). The scale bar represents 5 μm. See also Figure S3A.

(F) Immunofluorescence of quiescent cells with antibody to phospho-Aura (green), acetylated α-tubulin (blue), and DNA (red). The scale bar represents 12.5 μm.

CALK is very distantly related to the human Aurora A (AurA) kinase, with 55% similarity centered on the protein catalytic domain. In humans, Aurora A (AurA) is a centrosomal kinase that regulates mitotic entry through activation of Cdk1-cyclin B and other substrates that organize the mitotic spindle (Bischoff et al., 1998; Marumoto et al., 2005). AurA amplification or activation is common in many cancers characterized by centrosomal amplification and genomic instability (Anand et al., 2003; Goepfert et al., 2002; Gritsko et al., 2003). In the past 2 years, altered expression of the HEF1 (Law et al., 1996; O'Neill et al., 2000) scaffolding protein by amplification or epigenetic means has been identified as part of a prometastatic signature in breast cancer (Minn et al., 2005), shown to contribute to the aggressiveness of glioblastomas (Natarajan et al., 2006), and found to be critical for progression to metastasis in melanomas (Kim et al., 2006). Although HEF1 is best known as a transducer of integrin-initiated attachment, migration, and antiapoptotic signals at focal adhesions (O'Neill et al., 2000), we have recently documented interactions between HEF1 and AurA at the centrosome that are necessary for cellular progression through mitosis (Pugacheva and Golemis, 2005, 2006).

In this study, we demonstrate that an association between AurA and HEF1 at cilia in response to extracellular cues is required for ciliary disassembly. We also show that AurA activation is independently sufficient to induce rapid ciliary resorption, and that AurA acts in this process through phosphorylating HDAC6, thus stimulating HDAC6-dependent tubulin deacetylation (Hubbert et al., 2002) and destabilizing the ciliary axoneme. Importantly, our identification of a spatiotemporally restricted action of AurA at the ciliary basal body in cells emerging from G0 demonstrates an unexpected nonmitotic activity for AurA in vertebrate cells. We also determine that small molecule inhibitors of AurA and HDAC6 reduce regulated disassembly of cilia, which may have important implications for the action of these drugs in the clinic. Together, these data reveal important activities for HEF1, AurA, and HDAC6 in regulation of ciliary resorption, which should also inform the actions of these proteins in the cell cycle and cancer (Hideshima et al., 2005; Kim et al., 2006; Marumoto et al., 2005; Pugacheva and Golemis, 2005).

RESULTS

A System for Regulated Ciliary Assembly and Disassembly

We established a system to study ciliary dynamics in the hTERT-RPE1 cell line. 48 hr after plating cells at 50%–70% confluence in Opti-MEM medium without serum, >80% of hTERT-RPE1 cells had clearly visible cilia (Figure 1A). Cilia were typically of 3–4 μm length, with an acetylated α-tubulin-marked axoneme adjacent to two γ-tubulin-positive structures reflecting the basal body and the second cellular centriole (Figure S1A in the Supplemental Data available with this article online). Treatment of these ciliated cells with medium containing 10% fetal bovine serum (FBS) caused ciliary disassembly over the following 24 hr (Figure 1B). This disassembly occurred in two waves, with the first occurring 1–2 hr after serum stimulation and the second after 18–24 hr. FACS analysis, BrDU staining, and observation of condensed DNA and mitotic figures indicated that cells remained predominantly in G1 phase at 2 hr after serum addition, while during the 18–24 hr disassembly wave, most cells were entering mitosis (Figures S1B and S1C). This disassembly behavior was not unique to hTERT-RPE1 cells, as we observed a comparable biphasic resorption profile in the IMCD-3 murine and Caki-1 human renal cell lines (Figures S1D and S1E). To begin to assess serum components that might regulate ciliary disassembly, we have assessed PDGF, TGF-β, and EGF (Figure S2). Of these, only PDGF elicited a partial response. Full disassembly likely requires the combined input of several distinct serum components.

Dynamic Regulation of HEF1 and AurA at the Basal Body during Ciliary Disassembly

AurA (Figure 1C) and HEF1 (Figures 1D and 1E) localized to the basal body and the second centriole in quiescent, ciliated hTERT-RPE1 cells. In contrast, activated (T^{288}-phosphorylated) AurA was not detected at basal bodies of cilia in quiescent cells (Figure 1F and 1I [0 hr]) under fixation conditions at which it was clearly evident in mitotic cells (Figure 1G).

If AurA were functionally important for ciliary disassembly, we would expect changes in the activity of AurA 1–2 hr after serum treatment, potentially accompanied by changes in the AurA activator HEF1. Indeed, HEF1 expression increased at 1–2 hr after serum stimulation, dropped, and peaked again at ~18–24 hr after serum stimulation (Figure 1H). HEF1 initially appeared as a faster migrating 105 kDa species, with a slower migrating 115 kDa species appearing later. This 115 kDa species represents S/T-phosphorylated HEF1, is most abundant during the G2/M compartment in actively cycling cells, and is associated with AurA activation (Law et al., 1998; Pugacheva and Golemis, 2005). Total AurA levels sometimes increased slightly at 2 hr after serum stimulation, but were largely unaffected (Figures 1H). In contrast, peaks of

(G) Immunofluorescence of serum-stimulated cells with antibody to phospho-Aura (green), acetylated α-tubulin (blue), and DNA (red). The scale bar represents 5 μm.

(H) Western analysis of AurA and HEF1 in hTERT-RPE1 cells after serum stimulation. Western blots shown represent strips and reprobes of a single gel. Higher molecular weight HEF1 band reflects hyperphosphorylation, and coincides with AurA activation and ciliary disassembly at 2 and 24 hr after serum addition (at time point 0). Light gray arrow indicates crossreactivity of phospho-AurA directed antibody with total AurA; black arrow indicates phospho-AurA. See also Figure S1H.

(I) Immunofluorescence depicting AurA activation in serum-stimulated cells during disassembly of cilia. All images are merged panels of acetylated α-tubulin (red), phospho-AurA or total AurA (green) and DNA (blue).

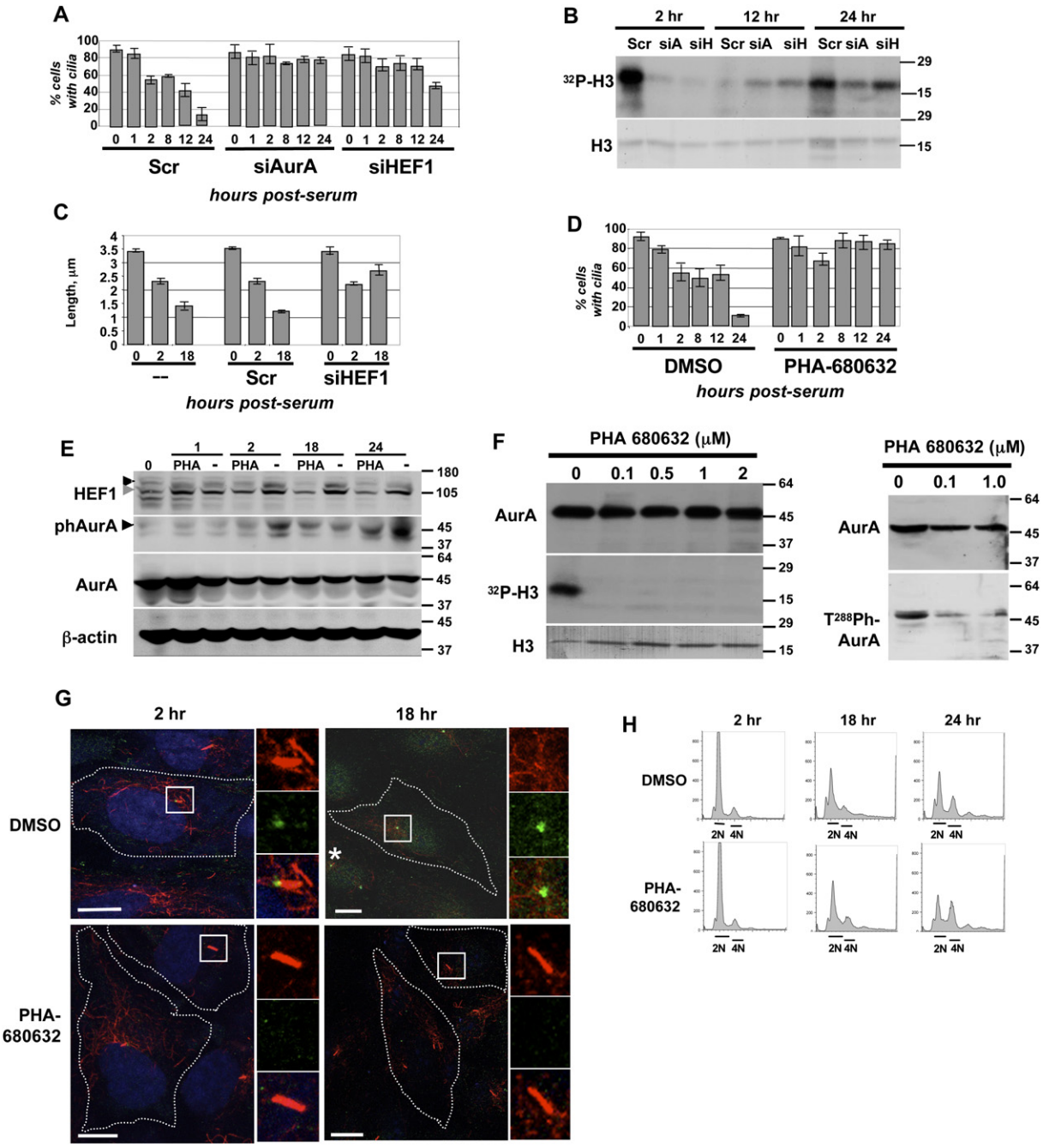

Figure 2. Activation of AurA Is Necessary for Ciliary Resorption

(A) Disassembly of cilia in cells treated with siRNA to AurA or HEF1, or with Scrambled (Scr) control siRNA, for 0 to 24 hr after serum addition. Assay performed three times, with an average of 100 cells counted/experiment by acetylated tubulin staining, error bars show SD. Results were confirmed using a second antibody (anti-glutamylated tubulin) to independently score cilia following depletion (Figures S4D and S4E).

(B) Ciliary disassembly was induced in ciliated cells pretreated with control (Scr), AurA-targeted (siA), or HEF1-targeted (siH) siRNA by supplementing growth media with serum. At 2, 12, and 24 hr after addition of serum, AurA was immunoprecipitated and used for an in vitro kinase assay as in (Pugacheva and Golemis, 2005). Shown, ^{32}P-labeled phosphorylated histone H3 (top) and total histone H3 in the reaction (stained with Coomassie Blue, bottom).

(C) Length of cilia in untreated hTERT-RPE1 cells (–), or the hTERT-RPE1 cells treated with control (Scr) or HEF1 targeting siRNA, at the indicated time points, error bars show the SD.

(D) Ciliated hTERT-RPE1 cells were treated with AurA inhibitor (PHA-680632) or DMSO, then disassembly of cilia tracked for 24 hr after serum addition, error bars show the SD. The in vitro IC50 of PHA-680632 is 27 nM for AurA; this compound also less potently inhibits AurC, AurB, and

phospho-T^{288}-AurA appeared precisely at each of the two waves of ciliary disassembly (Figures 1H and 1I). Strikingly, phospho-T^{288}-AurA was almost never detected at a basal body near a well-formed cilium. Although phospho-T^{288}-AurA invariably colocalized with both γ-tubulin-marked basal bodies/centrioles and with total AurA, in 85%–90% of cells with phospho-T^{288}-AurA, centrioles had no accompanying cilium. In 10%–15% of cells with phospho-T^{288}-AurA, centrioles with adjacent acetylated α-tubulin-marked cilia were observed, but these cilia were significantly shortened (~1–2 versus 3.5 μm) (Figure 1I). Similar profiles of HEF1 and AurA expression and activation were observed in serum-treated IMCD3 and Caki-1 cells, and PDGF-treated hTERT-RPE1 cells (Figures S2B, 2C, 2F, and 2G). The simplest interpretation of these results is that activation of AurA at the basal body immediately precedes the rapid disassembly of cilia.

HEF1-Dependent Activation of AurA Induces Ciliary Disassembly

We used two complementary approaches to establish that AurA activation is necessary and sufficient for induction of ciliary disassembly, and that HEF1 is likely to contribute to this process. First, exponentially growing hTERT-RPE1 cells were treated with siRNA targeting AurA or HEF1, or with control siRNA, plated for 2 days in OptiMEM to allow cilia formation, then treated with serum to induce ciliary disassembly. Immunoblotting confirmed siRNA treatment efficiently depleted AurA and HEF1 (Figure S4A). AurA depletion blocked and HEF1 depletion greatly limited serum-induced disassembly (Figure 2A). AurA activation was substantially reduced in cells treated with siRNA to HEF1 (Figure 2B); this correlated with reduced levels of AurA in HEF1-depleted cells (Figure S4B), implying HEF1 contributes to AurA stabilization as well as activation. Particularly at the second wave of ciliary disassembly, the residual cilia in HEF1-depleted cells were significantly longer than those in control cells (Figure 2C), implying that HEF1 modulates the disassembly process. Importantly, cells treated with siRNA to AurA or HEF1, or with control siRNA, were all ~80% ciliated before addition of serum, leading us to conclude that the predominant role for HEF1 and AurA is at the time of disassembly, i.e., these proteins are not required to form cilia.

Second, we used the small molecule AurA kinase inhibitor PHA-680632 (Nerviano Medical Sciences [Soncini et al., 2006]) to inactivate AurA kinase (Figures 2D and 2E). Disassembly of cilia was strongly reduced in cells pretreated for 2 hr with 500 nM PHA-680632 (Figure 2D). Although some ciliary disassembly was observed at 1 and 2 hr after serum stimulation, the percentage was lower than in DMSO-treated cells, and disassembly was not maintained, with cilia consistently re-established at the 8- and 12 hr time points. The second wave of ciliary disassembly, at the time of mitosis, was completely eliminated in PHA-680632-treated cells (Figure 2D). In cells with inhibited AurA, hyperphosphorylated HEF1 did not accumulate significantly at either wave of ciliary disassembly, indicating AurA dependence of this phosphorylation (Figure 2E). Western blot (Figures 2E and 2F, right panels), in vitro kinase assays (Figure 2F, left panels) and immunofluorescence (Figure 2G) confirmed the effectiveness of the compound in blocking AurA activation.

Together, these data imply that activation of AurA by HEF1 contributes to resorption of cilia at 2 and 18 hr following serum stimulation (Figures 2A–2E) and that active AurA is necessary to stably complete the disassembly process, but that HEF1 may not be the sole factor driving AurA activation and ciliary resorption (Figure 2A). Further, FACS analysis of cells with siRNA-depleted HEF1 or AurA (Figure S4C), or drug-inhibited AurA (Figure 2H) indicated that the blocked resorption of cilia at the 2 hr time point does not reflect an indirect consequence of altered cell cycle compartmentalization due to AurA inhibition. Cells indeed show predictable siRNA- and drug-induced accumulation in G2 at 18–24 hr after serum stimulation, which may account for the reduced resorption at these time points. However all cells at 2 hr after serum treatment have similar cell cycle profiles, remaining predominantly in G0/G1. Hence, the role of HEF1 and AurA at this early nonmitotic time point represents an unexpected direct action of these proteins.

AurA Activation Is Sufficient to Induce Rapid Disassembly of Cilia

Next, as a direct approach to establish sufficiency of active AurA to induce disassembly, we microinjected preactivated wild-type AurA (aAurA), T288A AurA (a hypomorphic mutant, (Satinover et al., 2004)), D274N AurA (an inactive mutant), GST, or buffer alone, together with fluorescent marker dye, into hTERT-RPE1 cells with preformed cilia. Microinjection of aAurA rapidly induced the disappearance of cilia from cells maintained in low serum medium: essentially as soon as cells could be fixed after microinjection,

FGFR1 (IC50 120, 185, and 390 nM, respectively, [Soncini et al., 2006]). Results were confirmed using anti-glutamylated tubulin, as shown in Figure S4D.

(E) Analysis performed in parallel with experiments described in D demonstrates PHA-680632 blocks appearance of T^{288}-phospho-AurA (visualized with antibody from BioLegend), and HEF1 phosphorylation (115 kDa form), in reference to DMSO (–) at the 2 and 24 hr time points. Black arrows marks phosphorylated AurA, and hyperphosphorylated (p115) HEF1; gray arrow indicates p105 HEF1. See also Figure S1I.

(F) Cells were treated with indicated concentrations of the AurA inhibitor PHA-680632, and then AurA immunoprecipitated, and used for in vitro kinase reactions (left) or whole cell lysates used for Western analysis with antibody to total or phosphorylated AurA (right).

(G) Immunofluorescence analysis of appearance of phospho-AurA at times indicated after serum stimulation in DMSO- or PHA-680632-treated cells. DNA (blue), acetylated α-tubulin (red), and T^{288}-phospho-AurA (green). In 18 hr DMSO/ph-AurA, an asterisk (*) marks a rare observation of phospho-AurA at the base of a shortened cilium.

(H) FACS analysis of cells treated with DMSO vehicle or PHA-680632 at the times indicated after serum stimulation.

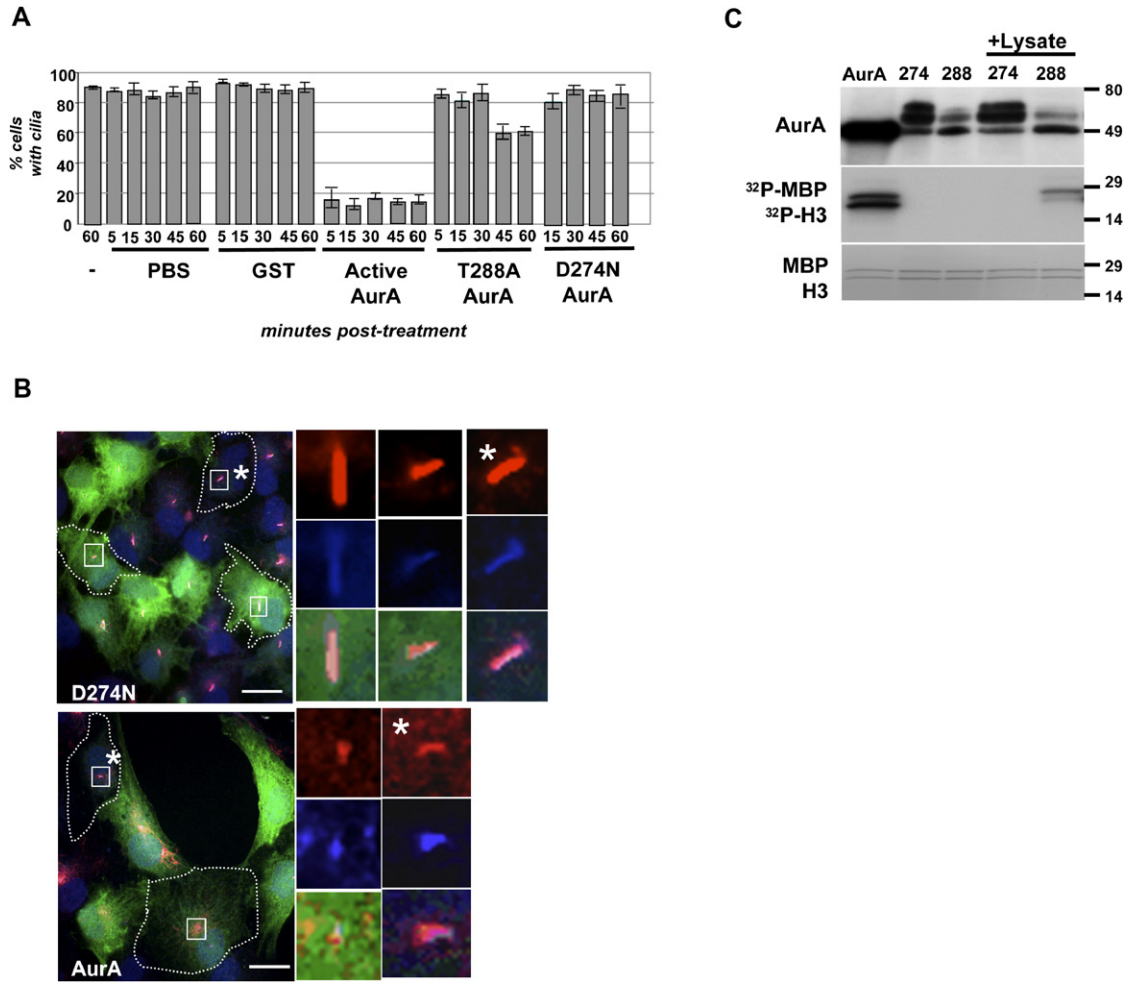

Figure 3. Microinjection of Active AurA Causes Rapid Loss of Cilia

(A) Microinjection of wild-type AurA, T288A or D274N mutant AurA, or GST, or PBS buffer, into hTERT-RPE1 cells with preformed cilia. (–), uninjected controls. Time reflects minutes from injection to initiating fixation of slides. Experiments repeated three times, with >100 injected cells scored in each experiment, error bars show SD.

(B) Cilia 45 min postinjection of AurA or D274N. Red, acetylated α-tubulin; blue, glutamylated α-tubulin (a second independent marker of cilia); blue, DNA; green, Dextran488 indicates injected cells. High magnification images to right are from boxed cells; an asterisk (*) marks magnification of uninjected cells.

(C) AurA and mutants (D274N, T288A) were incubated with histone H3 (17 kDa) and MBP (22 kDa) substrates in an in vitro kinase assay, confirming the activity of kinase. +Lysate indicates that mutants were incubated for 3 hr at 4°C with hTERT-RPE1 cell lysate, then pulled down and used for the kinase assay.

more than 80% of injected cells lacked cilia (Figures 3A and 3B). In contrast, injection of GST or buffer did not induce loss of cilia. Of the two mutants, D274N did not induce loss of cilia, while T288A caused eventual partial loss of cilia (Figure 3A) and ciliary shortening (results not shown). The ability of aAurA, T288A, and D274N paralleled the behavior of these proteins in in vitro kinase assays performed in parallel to microinjections (Figure 3C). Whereas aAurA was highly active and D274N was completely inactive, T288A became weakly active following brief incubation with cell lysates. Hence, the delayed resorption of cilia and ciliary shortening induced by T288A likely reflects the gradual emergence of an active pool of AurA following microinjection.

HDAC6 Is Required for Ciliary Disassembly

Little is known about the cellular machinery necessary for disassembling cilia. In seeking targets of AurA phosphorylation that might be relevant to this process, we considered the possibility that the acetylated α-tubulin commonly used to visualize cilia might play an active role in stabilizing the ciliary axoneme, based on reports that α-tubulin deacetylation promoted the in vivo destabilization of microtubules (Matsuyama et al., 2002). In particular,

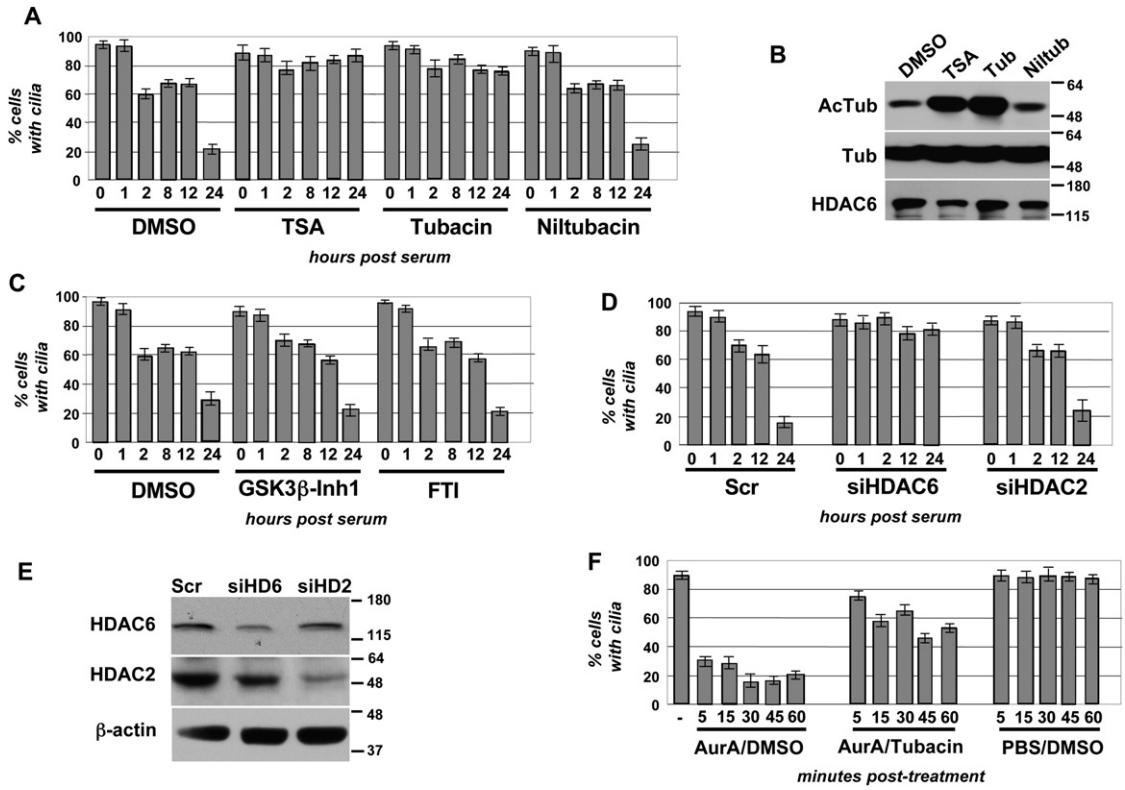

Figure 4. HDAC6 Activity Is Necessary for Resorption of Cilia

(A) Treatment of hTERT-RPE1 cells with histone deacetylase inhibitors prevents ciliary resorption. Cells were incubated with indicated compounds or vehicle (DMSO) at concentrations described in the Experimental Procedures for 2 hr prior to induction of ciliary disassembly. The assay was performed three times, with an average of 100 cells counted/time point.

(B) TSA and tubacin increase intracellular levels of acetylated tubulin. Shown, Western blot with indicated antibodies showing levels of acetylated tubulin in cells treated with TSA, tubacin, niltubacin, or vehicle (DMSO).

(C) GSK3β inhibitor and farnesyltransferase inhibitor (FTI) do not inhibit ciliary disassembly.

(D) Depletion of HDAC6 restricts serum-induced disassembly of cilia in hTERT-RPE1 cells transfected for 48 hr with siRNAs to HDAC6, HDAC2, or a scrambled control.

(E) Western analysis of hTERT-RPE1 cells treated with siRNA to HDAC6, HDAC2, or scrambled control.

(F) Active AurA or PBS were microinjected into hTERT-RPE1 cells pretreated for 2 hr with tubacin or DMSO. Error bars in panels A, C, D, and F show SD.

histone deacetylase 6 (HDAC6) has been identified as an important cytoplasmic tubulin deacetylase that influences mitosis and chemotaxis through regulating tubulin stability (Hubbert et al., 2002).

To assess whether altered regulation of tubulin acetylation might mediate HEF1/AurA signaling, we treated ciliated hTERT-RPE1 cells with small molecule deacetylase inhibitors, and established the ciliary disassembly profile (Figure 4A). Both the broad-spectrum HDAC inhibitor trichostatin A (TSA), and tubacin, an inhibitor specifically targeting HDAC6 (Hideshima et al., 2005), completely blocked serum-induced ciliary disassembly, whereas niltubacin, an inactive analog of tubacin, and vehicle alone had no effect. Levels of acetylated tubulin were measured in treated cells, confirming that these were increased in cells treated with TSA and tubacin, but not in cells treated with niltubacin or control vehicle (Figure 4B). As a control, because both AurA and HDAC inhibitors blocked ciliary disassembly, we considered the possibility that regulated ciliary disassembly might be generally sensitive to signaling inhibitors because of nonspecific toxicities. However, serum induced disassembly with a normal profile in cells treated with inhibitors of GSK-3β and farnesyltransferase (FTI), indicating that blocked ciliary disassembly was specific response to impaired AurA and HDAC6 signaling (Figure 4C).

To further confirm a specific requirement for HDAC6, we next established that cilia do not disassemble in serum-treated cells with siRNA-depleted HDAC6 (Figures 4D and 4E). Finally, we have microinjected aAurA into ciliated cells pretreated for 2 hr with tubacin (Figure 4F). Tubacin pretreatment substantially limited the ability of microinjected AurA to disassemble cilia. Initial disassembly was slower, and in some cases transient, with a significant percentage of injected cells re-forming cilia by 1 hr after injection. As for AurA, neither tubacin treatment nor siRNA to

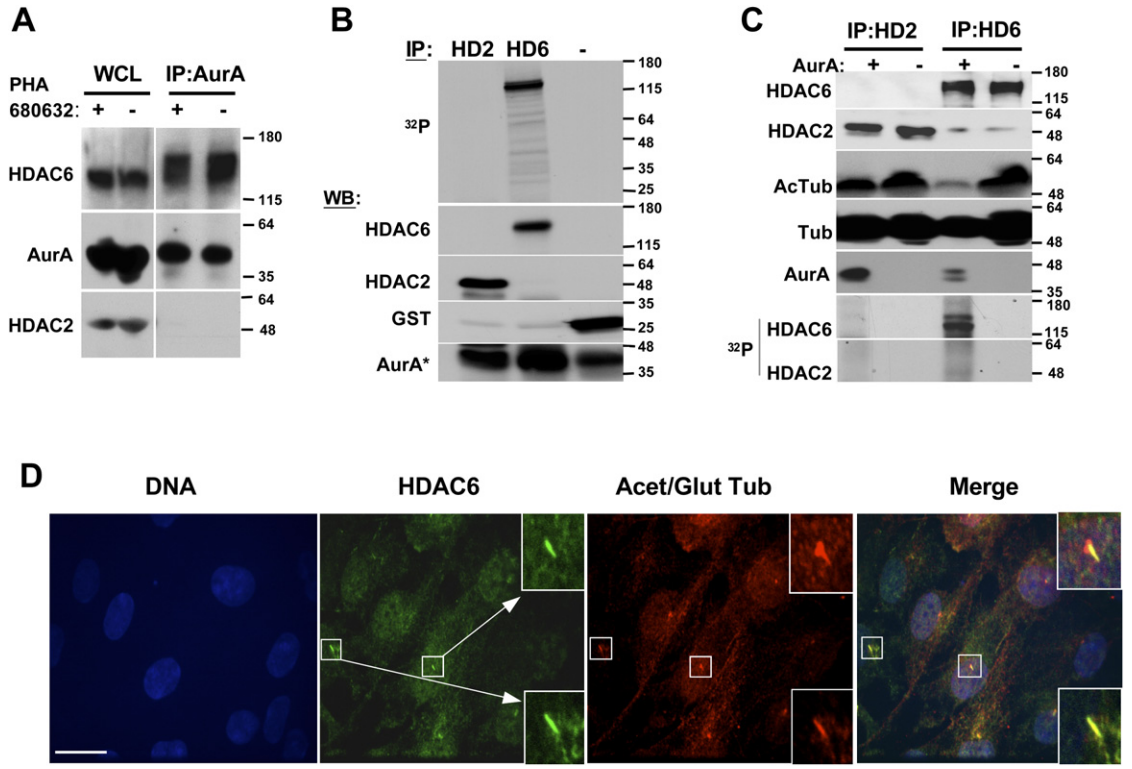

Figure 5. Direct Phosphorylation by AurA Activates HDAC6 Tubulin Deacetylase Activity

(A) hTERT-RPE1 whole-cell lysate (WCL) of cells treated with AurA inhibitor PHA 680632 (+) or with vehicle (−) was analyzed by Western blot directly, or following immunoprecipitation (IP) with antibody to AurA, using antibodies as indicated. The immunoprecipitation of the slow-migrating form of HDAC6 is not impacted by treatment of cells with PHA-680632, indicating that it most likely represents HDAC6 modified by an additional (unknown) cellular kinase/s.

(B) AurA phosphorylates HDAC6. In vitro translated and immunoprecipitated HDAC2 or HDAC6 (HD2, HD6), or recombinant GST (−), were mixed with recombinant AurA and used in an in vitro kinase assay. Reaction was split and used for autoradiography (^{32}P) or Western Blot (WB).

(C) In vitro translated HDAC2 or HDAC6 (HD2, HD6) were immunoprecipitated. IPs were mixed with AurA (+) or buffer (−), then used for either an in vitro tubulin deacetylation assay, or in an in vitro kinase assay using γ-^{32}P-ATP (see Experimental Procedures). Reaction mix was visualized by Western blot and by autoradiography, as indicated.

(D) HDAC6 localizes to disassembling cilia 2 hr after serum treatment. The scale bar represents 15 μM.

HDAC6 influenced cell cycle profile at 2 hr after serum stimulation, although both treatments led to accumulation in G2 at the later time point (Figures S4F and S4G). As a final control, we again used antibody to glutamylated tubulin as an independent means of scoring ciliary disassembly (Figure S4E). The results of these experiments are equivalent to those obtained using antibody to acetylated α-tubulin (Figures S5A–S5C). Based on these data, we concluded that HDAC6 is an important downstream AurA effector for ciliary disassembly.

AurA Phosphorylates HDAC6 to Activate Tubulin Deacetylase activity

Taken together, our data suggested that the mechanism of ciliary disassembly by AurA requires intact HDAC6 deacetylation activity, to destabilize microtubules. AurA-dependent regulation of tubulin deacetylation may be direct or indirect. Importantly, although microinjection of AurA induced loss of ciliary α-acetylated tubulin as cilia disassemble, the nonciliary α-acetylation of cytoplasmic microtubule networks were unaffected, suggesting a specific action of AurA and HDAC6 at the cilia (Figure S5C). Further supporting this idea, HDAC6 localized to cilia in serum-starved cells and during the ciliary disassembly process (Figure 5D and unpublished data), providing a ready target for AurA phosphorylation. Demonstrating a direct AurA-HDAC6 connection, antibody to AurA coimmunoprecipitated HDAC6 from hTERT-RPE1 cells (Figure 5A). AurA-HDAC6 coimmunoprecipitation was not eliminated by pretreatment of cells with PHA-680632, indicating that the association was not regulated by AurA activation status (Figure 5A).

To directly determine whether HDAC6 might be an AurA substrate, recombinant activated AurA was used in an in vitro kinase assay with purified HDAC6, HDAC2, or GST, as in (Pugacheva and Golemis, 2005). AurA phosphorylated HDAC6, but not HDAC2 or the GST negative control (Figure 5B). We next immunoprecipitated in vitro

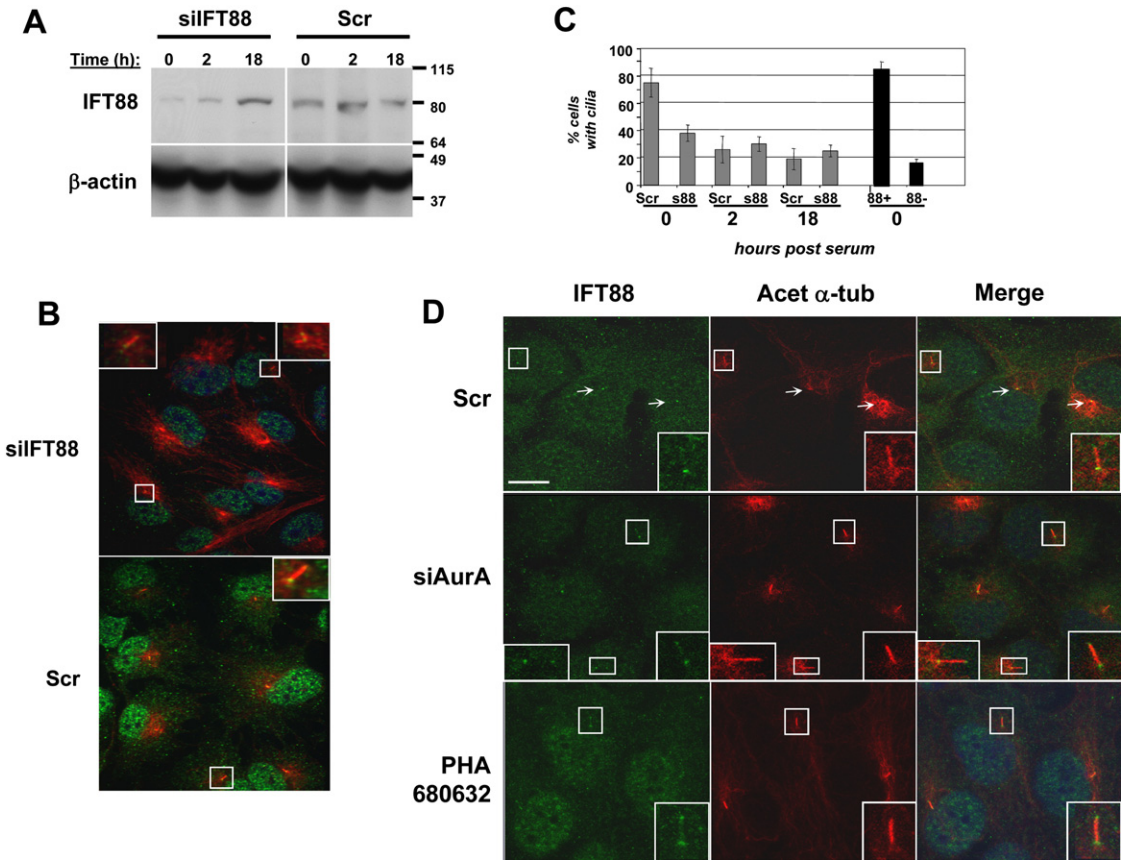

Figure 6. A Role for IFT Proteins in AurA-Induced Ciliary Resoprtion

(A) Western blot demonstrating siRNA depletion of IFT88 (siIFT88) in ciliated hTERT-RPE1 cells at times following serum treatment, relative to scramble-depleted control.

(B) Immunofluorescence matching Figure 6A at time 0, indicating relative degree of depletion of IFT88 at the basal body. Anti-acetylated α-tubulin, red; IFT88, green.

(C) Ciliary disassembly in IFT88- depleted (s88) versus Scr-depleted cells, at 0, 12, or 18 hr after serum treatment, based on the total cell population (gray bars). Black bars (right) indicate percent ciliated cells at time 0 calculated specifically from cells confirmed by immunofluorescence to have significant IFT88 staining (88+), or to be well-depleted for IFT88 (88−), error bars show SD.

(D) Cells treated with scrambled (Scr) or AurA-targeting (siAurA) siRNAs, or with PHA-680632 were fixed 2 hr after serum-initiated disassembly. Shown, immunofluorescence indicating cilia (anti-acetylated α-tubulin, red) and IFT88 (green). Insets are enlargements of boxed ciliary structures; arrows indicate cells without cilia. The scale bar represents 10 μm.

translated HDAC6 and a negative control, HDAC2, and gauged the relative ability of AurA to phosphorylate these proteins, and stimulate a tubulin deacetylase activity, in a defined in vitro assay. In reactions containing comparable levels of HDAC2 and HDAC6, only HDAC6 was phosphorylated by AurA (Figure 5C). Moreover, AurA-phosphorylated HDAC6 was much more potent than unphosphorylated HDAC6 in deacetylating α-tubulin (Figure 5C). These results lead us to conclude that AurA phosphorylation of HDAC6 stimulates HDAC6 deacetylase activity.

Ciliary Disassembly and Intraflagellar Transport

Intraflagellar transport (IFT) proteins perform important roles in mediating transport of proteins to and from the apical tip of cilia, and in many cases mutations in IFT proteins have been linked to ciliary dysfunction, loss of cilia, and pathological conditions (Sloboda, 2005). In contrast to depletion of HEF1 or AurA, depletion of representative IFT proteins IFT88 (Figures 6A–6C) and IFT20 (Figure S6) limits the initial formation of cilia in hTERT-RPE1 cells, similar to reports in other cell types (Follit et al., 2006; Pazour et al., 2000). Based on immunofluorescence, cilia were only observed in IFT-depleted cells that retain at least some detectable IFT protein (Figure 6C). This clear requirement of IFT proteins for ciliary assembly hinders the dissection of the contribution of these proteins in disassembly. However, intriguingly, the existing cilia in IFT88- or IFT20-depleted cells undergo minimal disassembly following serum stimulation, with the difference particularly noticeable at the early (2h) time point (Figures 6C, S6). Further, depletion or inhibition of AurA alters the localization of IFT88 during the ciliary disassembly process. In

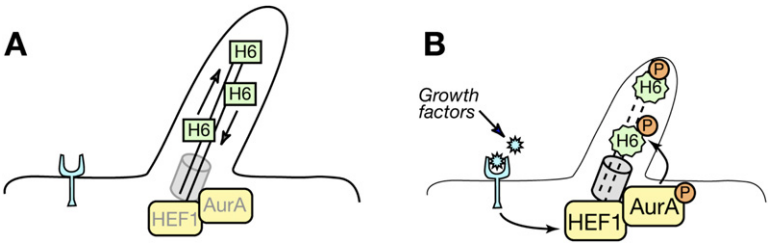

Figure 7. Working Model

(A) Aurora A (AurA) and low levels of HEF1 are localized to the basal body of quiescent, ciliated cells.

(B) Our data are consistent with a model in which growth factors induce HEF1 expression, promoting HEF1-dependent activation of Aurora A. This results in phosphorylation of ciliary HDAC6 6 (H6) by Aurora A, thereby inducing ciliary resorption.

untreated cells, IFT88 is seen intensely at the basal body and more diffusely along the axoneme of residual cilia two hours after serum stimulation, whereas in cells lacking active AurA, IFT88 accumulates at both the basal body and apical tip at this time point (Figure 6D). It is likely that as in *Chlamydomonas* (Pan and Snell, 2005), IFT signaling mediates some aspects of ciliary disassembly.

DISCUSSION

Cilia and flagella have been described as cellular ''antennas'', sensing a multiplicity of extracellular stimuli to induce an intracellular response (Singla and Reiter, 2006). In addition to undergoing regulated resorption induced by extracellular cues, for over four decades cilia have been known to be dynamically resorbed and resynthesized throughout the cell cycle. Taken in sum, our data suggest a model (Figure 7) in which the serum growth factor-induced activation of a HEF1-AurA complex allows AurA to phosphorylate and activate HDAC6, which destabilizes the ciliary axoneme by deacetylating tubulin. Unexpectedly, activation of AurA is a central component of this cascade even during the G1 resorption wave, indicating a nonmitotic activity for AurA in animals.

An important finding of this work is the novel connection between AurA and HDAC6. HDAC6 tightly interacts with α and β tubulins through its HDAC domain, which may restrict its enzymatic activity, based on reports that taxol treatment causes HDAC6 to accumulate on microtubules, and is accompanied by increased tubulin acetylation (Zhang et al., 2003). Localized phosphorylation by AurA may increase the turnover of HDAC6 at microtubules, thus increasing the active pool of HDAC6 at cilia. Interestingly, studies in *Chlamydomonas* indicate that an important element of flagellar resorption is destabilization of the microtubule-based axoneme, suggesting this signaling cascade may be evolutionarily conserved (Pan and Snell, 2005; Pan et al., 2004). Further supporting the idea of conservation, the *C. elegans* gene MEC-12 encodes an α-tubulin variant that is specifically required only in mechanosensing neurons, which depend on intact cilia: MEC-12 is the only α-tubulin in this species with a conserved site for acetylation (Fukushige et al., 1999). Interestingly, HDAC6 has been reported to associate with protein phosphatase 1 (PP1) (Brush et al., 2004), which binds microtubules (Liao et al., 1998), and dephosphorylates and inactivates AurA kinase. Such feedback may limit AurA activation at cilia.

A number of growth stimuli induce HEF1 expression and phosphorylation, influencing its protein interactions. These include PDGF, which is here shown to partially induce ciliary disassembly (Natarajan et al., 2006). Intriguingly, recent studies of p130Cas, a protein structurally similar to HEF1, indicate that p130Cas acts as a stretch sensor; HEF1 contains all sequence motifs necessary for similar function (Kostic and Sheetz, 2006). As one major function of cilium is to sense fluid flow, and overly persistent flow has been reported to induce ciliary disassembly (Iomini et al., 2004), stretch sensation may be an important action of HEF1. Our data suggest that HEF1 both activates AurA and stabilizes the protein from degradation; it will be interesting to determine if the HEF1 scaffolding activity also contributes to AurA interaction with its effector HDAC6. Our data also indicate that AurA activity influences IFT88 localization during disassembly, and suggest integrity of the IFT system is important for the disassembly process in animals, as in *Chlamydomonas* (Pan and Snell, 2005).

Our establishment of a HEF1-AurA-HDAC6 cascade at cilia also informs the understanding of the mitotic activities of these proteins. Dynamic changes in microtubule acetylation and deacetylation characterize the stages of mitosis, and HDAC inhibitors that inhibit family members with microtubule deacetylase activity induce mitotic arrest (Blagosklonny et al., 2002). The identification here of HDAC6 as an AurA target suggests that HEF1-AurA regulation of tubulin deacetylation at mitosis through HDAC6 might offer a mechanism to fine-tune the mechanical properties of the mitotic spindle. This signaling cascade may also influence re-establishment of focal adhesions at and following cytokinesis, given the growing appreciation of the role of microtubules in guiding the formation of these structures (Ezratty et al., 2005; Strickland et al., 2005). Further, one intriguing possibility is that the common use of an AurA-HEF1-HDAC6 switch at the basal body of quiescent cells and the centrosome of G2/M cells may serve as part of a checkpoint mechanism coordinating responsiveness to extracellular cues at different points in cell cycle. In this context, our observation that inhibition of AurA causes appearance of mitotically arrested cells possessing both spindles and cilia (results not shown) may reflect triggering of such a centrosomally based checkpoint.

These results also have implications for the understanding and treatment of cancer. Tumor cells commonly do not have cilia, and both HEF1 and AurA are often upregulated in cancer. The roles for these proteins at the centrosome

and focal adhesions described earlier already offer two mechanisms by which these proteins may promote tumor initiation and progression. The current study indicates a third mechanism, in which elevation of HEF1 or AurA in tumors may destabilize cilia, thus conditioning cellular response to external cues and impacting multiple signaling pathways. Further, AurA is regarded as a promising chemotherapeutic target, with agents inhibiting this protein currently in clinical trials (Andrews, 2005). TSA and other broad-spectrum agents targeting HDACs are used in the clinic (Vanhaecke et al., 2004), with more focused agents such as tubacin in preclinical development (Hideshima et al., 2005). Our data suggest that AurA- or HDAC-targeted drugs may have previously unappreciated in vivo effects involving cilia, that may contribute to the observed efficacy and/or side effects of these agents.

PKD is one of the best-described cilia-related diseases (Wilson, 2001), with mutation of the cilia-localized polycystin proteins 1 and 2 (PKD1 and PKD2) responsible for the significant majority of PKD patients. p130Cas interacts directly with complexes containing PKD1 and PKD2, and also with nephrocystins, cilia-associated proteins that are mutated in a second renal cystic syndrome, nephronophthisis (Benzing et al., 2001). Although an association of HEF1 with these proteins has never been assessed, HEF1 is abundant in the kidney and conserves many protein interaction sequences with p130Cas. It is also tantalizing to consider that closer connections exist between dysplastic disorders leading to cysts and cancer than have previously been appreciated. One of the surprising results of a recent large study to analyze the cancer genome was the identification of the PKHD1 protein, a ciliary protein which is mutant in autosomal recessive PKD, as commonly mutated in colorectal cancer (Sjoblom et al., 2006). Overall, deregulated AurA/HEF1/HDAC6 signaling may have broad implications for studies of human development and disease.

EXPERIMENTAL PROCEDURES

Cell Culture and siRNA

hTERT-RPE1 cells were grown in DMEM with 10% fetal bovine serum (FBS). For analysis of ciliary disassembly, cells were plated at 30% confluence in plates containing glass cover slips, and starved for 48 hr (in Opti-MEM or regular DMEM, without added serum) to induce cilia formation, followed by treatments described in Results. Details of siRNAs used for depletion of HEF1, AurA, HDAC6, HDAC2, IFT88, IFT20, and control siRNAs, are available on request. For siRNA treatment, cells were initially plated in DMEM/10%FBS in plates containing cover slips, and 12 hr later siRNA transfection was performed in Opti-MEM with Oligofectamine (Invitrogen) according to manufacturer recommendations, and fixed 48 hr after transfection, following treatments indicated in Results. The remaining cells on plate were lysed, then either directly analyzed by Western blot analysis, or used for immunoprecipitation (IP)-kinase reaction to measure AurA activity.

Drug Inhibition Experiments

The Aurora kinase inhibitor PHA-680632, GSK3β-inhibitor 1 (Calbiochem), FTI-277 (Calbiochem), Tubacin, Niltubacin or DMSO vehicle were added to hTERT-RPE1 cells 2 hr prior to the initiation of ciliary disassembly. After initial titration experiments to establish effective range, PHA-680632 was used at 0.5 μM, Tubacin and Niltubacin at 2 μM, GSK3β-inhibitor 1 at 2 μM, FTI-277 at 50nM concentration for the experiments described.

Protein Expression, Western Blotting, and Immunoprecipitation

For microinjection, recombinant glutathione-S-transferase (GST), GST fused AurA mutants T288A and D274N) produced from BL21 (DE3) bacteria were purified using the MicroSpin GST Purification Module (Amersham Biotech.). Purified recombinant AurA was purchased from Upstate; this AurA was preactivated based on incubation with ATP. Mutationally inactive AurA (T288A,) was also made using a baculoviral expression system (Invitrogen), and was purified by Ni-Sepharose 6FF (Amersham).

To prepare lysates for Western blotting and IP, mammalian cells were disrupted by M-PER lysis buffer (Pierce) supplemented with EDTA-free protease inhibitor cocktail (Roche). Lysates used for IP were incubated overnight with antibody at 4°C, subsequently incubated for 2 hr with protein A/G-sepharose (Pierce), washed, and resolved by SDS-PAGE. Western blotting was performed using standard procedures and proteins visualized using the West-Pico system (Pierce). Antibodies used included mouse monoclonal antibody (mAb) anti-HEF1 2G9 (Pugacheva and Golemis, 2005), anti-α-tubulin mAb (Sigma), anti-AurA (BD Bioscience) for Western blotting, anti-AurA rabbit polyclonal (Cell Signaling) for IP, anti-Phospho-AurA/T288 (BioLegend), anti-Phospho-AurA/T288 (Cell Signaling), anti-HDAC6 rabbit polyclonal (Upstate; 1:5000), anti-HDAC2 rabbit polyclonal (Invitrogen) and mAb anti-β-actin (AC15, Sigma), anti-IFT88 and anti-IFT20. Secondary horseradish peroxidase (HRP)-conjugated antibodies were from Amersham Biotech.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (10 min) then methanol (5 min), permeabilized with 1%Triton-X100 in PBS, blocked in 1× PBS, 3% BSA, and incubated with antibodies using standard protocols. Primary antibodies included rabbit polyclonal anti-Aurora A and anti-phospho-AuroraA/T288, (Cell Signaling), mouse mAb anti-HEF1 (14A11), polyclonal anti-γ-tubulin (Sigma), anti-α-tubulin mAb (Sigma), anti-acetylated α-tubulin mAb (clone 6-11B-1, Sigma, and clone K(Ac)40 Biomol), anti-IFT88 and anti-IFT20 (gifts of G. Pazour), mouse anti-glutamylated tubulin (Sigma), and anti-HDAC6 (Upstate). Secondary antibodies labeled with Alexa-488, Alexa-568, and Alexa-633, and TOTO-3 dye to stain DNA, were from Molecular Probes/ Invitrogen. DNA was costained in some experiments by propidium iodine (Sigma) or Draq5 (Alexis). Confocal microscopy was performed using a Radiance 2000 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) coupled to a Nikon Eclipse E800 upright microscope (Nikon). Statistical analysis of data by one-way ANOVA was performed using GraphPad Instat 3.0 (San Diego, CA).

Microinjection

Microinjections were performed on a Nikon TE300 Microscope (Nikon, Melville, NY) that was equipped with an Eppendorf Transjector 5246 semiautomatic microinjector and micromanipulator (Eppendorf, Westbury, NY). Cells were plated on gridded coverslips (Belco) and starved for 48 hr before cytoplasmic microinjection of 0.05μM preactivated AurA (Upstate), inactive AurA (T288A) and (D274N), GST protein, or buffer. Proteins were prefiltered through a 0.2 μm Millipore membrane and mixed with Dextran Green488 (Molecular Probes) to mark injected cells. Injected cells were incubated at 37°C before fixation. Typically, 150 cells were microinjected in each of 3 experiments.

Kinase and Tubulin Deacetylation Assays

In vitro kinase assays were performed using recombinant active AurA (Upstate), mutationally inactive AurA purified from baculovirus and BL21(DE3) bacteria, or endogenous AurA immunoprecipitated from mammalian cells. A standard kinase reaction with γ-^{32}P(ATP) and histone H3 and MBP (Upstate) substrates was done as in (Pugacheva and

Golemis, 2005). For deacetylase assays, HDAC6 and HDAC2 were in vitro translated using a TnT-Coupled Reticulocyte Lysate System (Promega), immunoprecipitated, and incubated with/without active AurA(Upstate) in the presence of (25μg) stabilized microtubules prepared from purified bovine brain tubulin (Cytoskeleton) to measure deacetylase activity (as in (Hubbert et al., 2002)) and with γ-^{32}P-ATP (Perkin-Elmer) in AurA reaction buffer. 1/10 volume of samples were reserved for Western blotting.

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/7/1351/DC1/.

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Review

A New Central Scaffold for Metastasis: Parsing HEF1/Cas-L/NEDD9

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Abstract

Greater understanding of metastasis is required to improve cancer treatment outcomes. Recently, changes in expression of the scaffold protein HEF1/CAS-L/NEDD9 were found to be a potent prometastatic stimulus in melanoma and other cancers. Mechanistic studies suggest diverse cellular roles of HEF1 and highlight its importance in the response to extracellular cues that drive invasion and metastasis. As a metastatic "hub" for signaling in cancer, HEF1 may provide a useful target for drug discovery efforts. [Cancer Res 2007;67(19):8975–9]

Introduction

In recent years, our understanding of the metastatic process has evolved significantly. It is now appreciated that the formation of distant metastases requires functionally distinct events, including (*a*) invasion of a tumor through basement membrane and stroma, (*b*) tumor cell intravasation, (*c*) survival of a tumor in the blood stream, (*d*) homing of the tumor to a specific site (frequently, although not invariably), and (*e*) extravasation and colony formation. This complexity implies either that the combined action of multiple proteins is necessary for metastasis, or that proteins which can promote metastasis are multifunctional.

The identification of tumor-encoded genes, whose elevated expression marks transition to metastasis and which are required for establishment of tumors at distant sites, will yield important diagnostic and therapeutic benefits. Many research groups have sought to define such genes, and in the past year, signaling activity and overexpression of HEF1/Cas-L/NEDD9 (hereafter designated HEF1; refs. 1–3) has been shown to be required for invasion by glioblastomas (4) and strongly linked to promotion of melanoma metastasis (5). Significantly, extensive characterization of HEF1 over the past decade has revealed a multifaceted mode of action that suggests changes in HEF1 status may be fundamentally important for multiple aspects of the metastatic program. In this review, we will first discuss the signaling activities that most likely underlie the ability of HEF1 to influence metastasis and then outline the cellular mechanisms influencing HEF1 expression and "activation" during metastatic transition. Finally, we will discuss intriguing recent findings that imply a broader role for HEF1 in the pathogenesis of aggressive nonsolid tumors and strategies for therapeutically targeting HEF1. We note that roles for HEF1 in additional cellular processes and diseases have recently been comprehensively reviewed (6, 7).

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Biological Activities of HEF1 that Favor Metastasis

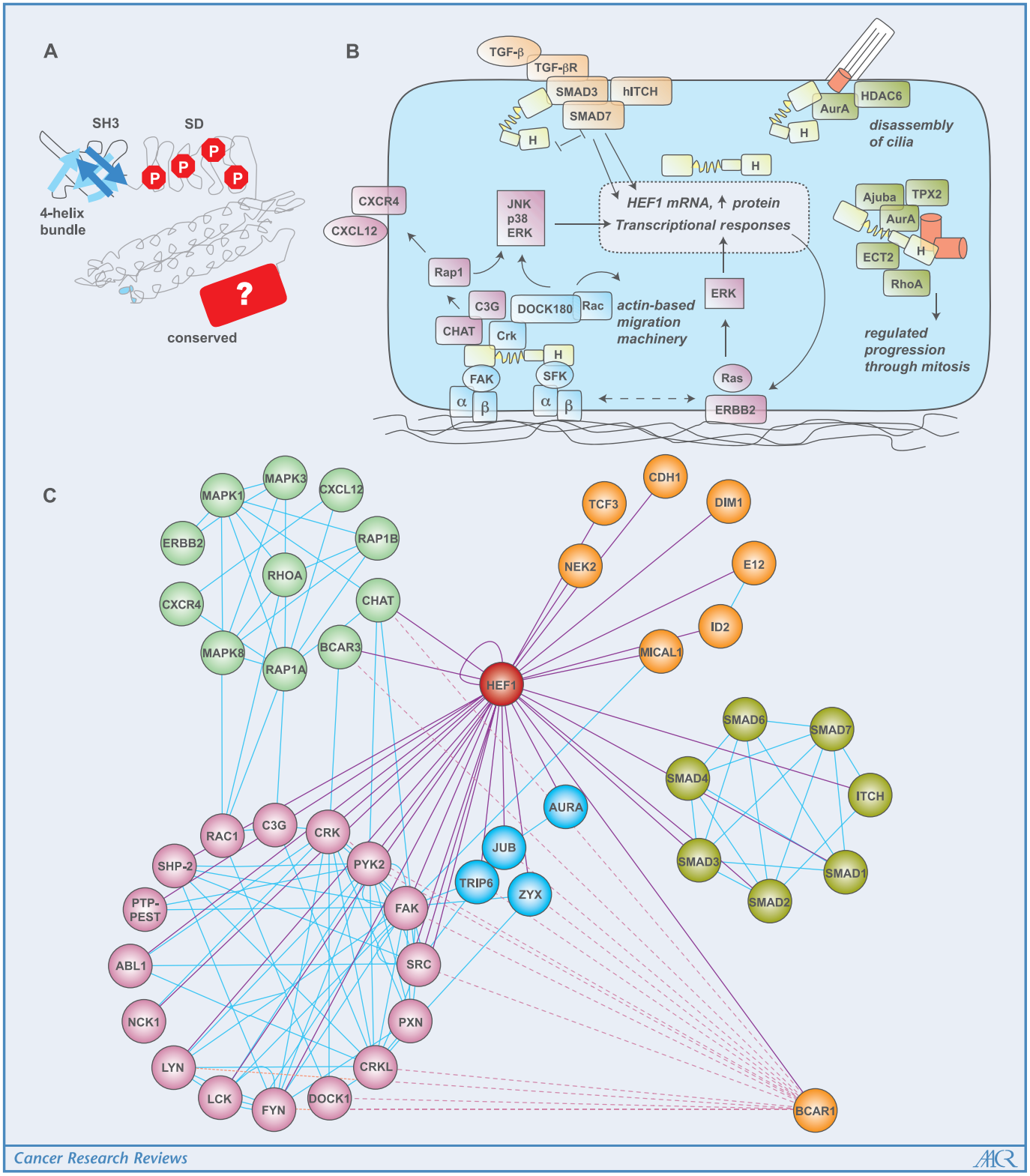
The *HEF1* gene is conserved in all vertebrates and localizes at chromosome 6p25-24 in humans and chromosome 13 A3.3 in mice. The HEF1 protein is predominantly cytoplasmic, concentrating at focal adhesions during interphase in adherent cells and at centrosomes and other parts of the mitotic apparatus during G_2-M. HEF1 lacks any known enzymatic function but contains many functional modules for protein interaction, leading to its classification as a scaffolding protein (2, 3, 7, 8). Validated interaction sequences include (Fig. 1*A*) an SH3 domain, at least 15 SH2 domain-binding sites, and an evolutionarily well-conserved carboxy-terminal domain of unknown structure. Proteins that functionally and/or physically interact with HEF1 (Fig. 1*B* and *C*) include many with direct roles in promoting tumor invasion, as discussed below. Vertebrate HEF1 has two paralogues, p130Cas/BCAR1 and Efs/Sin, which conserve domain structure and many, but not, all functional interactions (6, 8): together, HEF1, p130Cas, and Efs are called members of the Cas family.

Significantly elevated levels of HEF1 mRNA (10-fold to 70-fold) and protein exist in >35% of the metastatic melanomas produced by "escaper" tumors in *Tyr−rtTA+;Tet−RAS+;Ink4a/Arf−/−* mutant mice, as well as in a similar percentage of human metastatic melanomas (5). Importantly, elevated HEF1 protein expression was essential for the metastatic properties of the involved tumors both *in vitro* and *in vivo* (5). As HEF1 is a scaffolding protein, its action involves regulated assembly of protein complexes. Consequently, the effect of altered HEF1 expression is dependent on the relative stoichiometry and availability of other complex constituents. Hence, moderate overexpression may drive the assembly of functional complexes, causing constitutive activation of downstream effector pathways. Conversely, excessive HEF1 overexpression may be equivalent to loss of HEF1 expression, if either condition induces complex disruption. It is perhaps significant that one study has identified reduced HEF1 expression as part of a signature for metastatic breast cancers (discussed further below; ref. 9). Further, both overexpression and depletion of HEF1 cause mitotic defects in cultured cells (10, 11). Whereas the exact mechanism of HEF1 action in metastasis requires further investigation, to date, studies of HEF1 overexpression, depletion, and genetic deletion have revealed the following metastasis-relevant properties.

(*a*) HEF1 positively regulates the Src-FAK-Crk "migratory switch." The initial reports identifying HEF1 established that this protein interacts directly with FAK, Src, and Crk (2, 3). FAK is commonly constitutively activated in melanomas and an important target of cancer drug development. The consensus of work by many groups suggests a mechanism in which cell attachment triggers the interaction of Src, HEF1, and FAK: overexpression or mutational activation of one of these proteins can also drive complex formation. These interactions enhance the activation of Src and FAK and lead to extensive tyrosine phosphorylation of HEF1, creating binding sites for effector proteins with SH2

domains, the most important of which are Crk and Crk-L. A Cas-Crk complex has been described as a "master switch" for cell migration; through Crk-L and DOCK180, HEF1 activates Rac and other components of the cell migration machinery (reviewed in ref. 8).

(*b*) HEF1 activates machinery for tumor growth, invasion, and homing. Tumor progression depends on the activation of essential effector kinases that mediate proliferation and survival in an expanding hypoxic tumor mass. Tumor invasion depends on the increased expression and activity of extracellular proteases that



degrade or remodel basement membranes to allow cellular movement while activating latent growth factors that promote tumor progression. Related to this, HEF1 overexpression may induce molecules involved in tissue remodeling and invasion (12). HEF1 overexpression also induces the activation of extracellular signal-regulated kinase (ERK), p38, and c-Jun-NH_2-kinase (JNK) kinases through interactions with intermediary signaling effectors (reviewed in ref. 6). For example, overexpressed HEF1 transcriptionally induces ErbB2/HER2/neu, a growth factor receptor at the top of the Ras > Raf > MAP/ERK kinase > ERK pathway, whereas the HEF1 interactor FAK directly binds SHC, which influences activation of Ras through its binding partners, epidermal growth factor receptor and growth factor receptor binding protein 2.

HEF1 also binds proteins of the AND-34/CHAT family (13), which activate JNK and ERK by signaling through Rap1 (14). Activated Rap1 is also an important intermediate in "inside-out" cell signaling, in which internally derived signals activate integrin-ligand binding in response to upstream cues that typically involve chemokine stimulation. This process, much studied in consideration of the migration and "homing" of lymphoid cells, is now appreciated as playing a role in tumor invasion and targeting of metastases. Lymphoid cells from HEF1 null mice, or with depleted HEF1, are greatly impaired for chemokine response, migration, and homing, accompanied by failed activation of CHAT-H and Rap1 (14, 15). Critically, these defects involve the CXCR4-CXCL12 targeting system, which is important not only for targeting of lymphocytes to secondary organs, but is also a major contributor to tumor metastasis (16). By inference, CXCR4 signaling is expected to be hyperactivated in cells with overexpressed HEF1.

(*c*) HEF1 conditions transforming growth factor-β (TGF-β) responses. One of the enigmas of tumor progression is how tumor interpretation of TGF-β signals modulates over time. Extrinsic TGF-β inhibits the growth of early tumors; however, TGF-β promotes the growth of later stage invasive tumors (17), down-regulating E-cadherin and promoting mesenchymal transformation. Intriguingly, HEF1 binds directly to TGF-β pathway effectors and inhibitors, including multiple SMADs (e.g., ref. 18 and discussed in ref. 6). Via these interactions, HEF1 induces negative feedback for aspects of TGF-β–dependent signaling. Intriguingly, the TGF-β pathway signaling molecule SMAD7 has very recently been shown to inhibit melanoma metastasis to bone (19); HEF1 overexpression would be predicted to limit SMAD7 activity, thus promoting metastasis.

(*d*) HEF1 activates RhoA and Aurora A, providing early counter pressures for tumor growth. Given the extensive biology linking HEF1 to invasion signaling pathways, recent observations that this protein also regulates cell cycle progression through mitosis were unexpected (6, 10, 11). In MCF-7 cells and other epithelial cell lines, HEF1 functions at two discrete points during cytokinesis. At the centrosome, HEF1 interacts with and activates Aurora A kinase during mitotic entry. Overexpressed HEF1 hyperactivates Aurora A, inducing failure of cytokinesis. Separately, HEF1 positively regulates RhoA activation, and elevated HEF1 expression leads to abnormally persistent RhoA activity throughout cytokinesis, preventing normal cellular reattachment to surrounding matrix and providing a second stimulus for deficient cytokinesis. HEF1-overexpressing cells exhibiting defective cytokinesis then arrest in G_1 and subsequently undergo apoptosis at high frequency, implying the triggering of cell division checkpoints.

The recognition that HEF1 overexpression triggers cell division checkpoints and apoptosis may be particularly important in understanding why HEF1 overexpression is associated with later (rather than early) stages of tumor progression. In the *Tyr−rtTA+;Tet-RAS+;Ink4a/Arf−/−* melanoma mouse model, both Rb-dependent and p53-dependent cell division checkpoints have been disabled, and concurrently, constitutive Ras overexpression provides a strong stimulus toward continued proliferation. Such prior changes may be essential for cells to tolerate proapoptotic effects of sustained HEF1 overexpression. In this context, it is interesting that metastatic melanomas are often characterized by genomic rearrangements and aneuploidy and manifest a high level of apoptosis relative to premetastatic tumors.

(*e*) HEF1 and Aurora A regulate ciliary disassembly. Cilia are small organelles that protrude from the surface of many mammalian cell types and act as cellular "antennas," with growth factor receptors localized at cilia sensing extracellular cues to regulate cell growth. Defects in ciliary structural integrity or associated signaling induce numerous developmental syndromes, are a primary cause of polycystic kidney disease, and have been, in the past year, strongly linked to cancer development (reviewed in ref. 20). Very recently, HEF1 activation of Aurora A at the ciliary basal body was shown to trigger a ciliary resorption pathway involving the tubulin deacetylase HDAC6 as an effector protein (21). This unexpected finding suggests a totally new mechanism by which overexpression of HEF1 can influence the growth properties of metastatic cancers: much more work is necessary to understand the importance of this observation.

What Causes HEF1 Up-regulation before Melanoma Metastasis?

In normal and transformed cells, HEF1 is dynamically regulated in response to both intracellular and extracellular signals. Molecular mechanisms important for control of HEF1 expression

Figure 1. *A,* schematic of the structure of the HEF1/NEDD9/CasL protein. Human HEF1 is 834 amino acids; key functional domains include an amino terminal SH3 domains which binds FAK, a "substrate domain" (*SD*) containing multiple embedded SH2 binding sites analogous to the mechanosensing domain of p130Cas recently described by Sawada et al., a likely four-helix bundle based on molecular modeling of primary sequence of HEF1 against the crystal coordinates for the conserved region of p130Cas (results not shown), and an evolutionarily conserved C-terminal domain which binds Src family kinases and other proteins (indicated with a "?" as details of structure remain unknown). *B,* HEF1 (*bright yellow*, with "H") is a component of the integrin-dependent Src-FAK-Crk migration signaling cascade, influences cellular homing through CHAT/CXCR4, engages in crosstalk with the Ras pathway, is an intermediate in TGF-β–dependent signaling, activates the centrosomal Aurora-A/Ajuba/TPX2 machinery governing mitotic entry and cytokinesis, and activates Aurora-A/HDAC6 at the basal body to initiate ciliary disassembly. *C,* curated online resources (based on experimentally well-validated protein interaction data) indicate numerous direct HEF1 interactions with cancer-related signaling pathways. *Blue lines,* protein interactions; many HEF1 partners also take part in extensive self-interactions within functional clusters that are likely augmented by increased HEF1 levels. Clusters are particularly relevant to FAK/Src/integrin (*rose*), CHAT/Rap1 (*green*), Aurora A (*blue*), TGF-β/SMADs (*gold*), and all others (*yellow*). All HEF1-only interactions present in online databases (*blue lines*) and common interactions of HEF1 and BCAR1/p130Cas (*dashed pink lines*, for contrast) are distinguished; interactions between all other proteins, including functional and physical interactions (*green*). For clarity, only interactions relevant to the discussion in this review are shown. Data on protein-protein interactions were collected in Cytoscape (http://cytoscape.org/), combining data from a Bionet plug-in (http://err.bio.nyu.edu/cytoscape/bionetbuilder/), and EMBL String (string.embl.de/), with each retrieving information from several databases, including DIP (http://dip.doe-mbi.ucla.edu/), BIND (http://www.bind.ca/), KEGG (http://www.genome.jp/kegg/), Prolinks (http://mysql5.mbi.ucla.edu/cgi-bin/functionator/pronav), HPRD (http://www.hprd.org/), and The BioGrid (http://www.thebiogrid.org/).

and scaffolding function include transcriptional activation, phosphorylation, and both proteasome-mediated and caspase-mediated proteolysis (reviewed in detail in ref. 6). Although up-regulation of the HEF1 mRNA in metastasis has been shown in some cases to arise from chromosomal amplification (5), this up-regulation more commonly occurs at the level of mRNA transcription. Given that the HEF1 mRNA is down-regulated during nervous system development (ref. 1 and discussed in ref. 6), it is interesting to speculate that HEF1 overexpression in melanoma may reflect aberrant reactivation of a developmental program.

A growing number of transcriptional pathways have been reported to regulate HEF1; intriguingly, some of these are particularly relevant to metastasis. For example, hypoxia is now appreciated as creating conditions conducive for metastasis, and hypoxia is emerging as a potentially important inducer of HEF1 mRNA expression (22, 23). TGF-β strongly induces transcription of HEF1 (24); together with the role for HEF1 in negative regulation of TGF-β signaling, this provides a second link between these proteins and raises the possibility of a reinforcing feedback loop. HEF1 mRNA and protein are strongly induced by serum treatment, but as yet, most of the serum factors directing transcription of the HEF1 are poorly understood.

It is extremely likely that posttranscriptional events will be found to modulate the expression and action of HEF1 in aggressive cancers. Phosphorylation not only regulates HEF1 scaffolding capacity but can also regulate HEF1 protein turnover. HEF1 phosphorylation is induced by a number of key factors linked with cancer progression, including FAK (commonly activated in advanced melanomas), TGF-β (24), platelet-derived growth factor (4), Abl (2), and BCR-ABL (25). This last observation is of particular interest as the literature describing a role for HEF1 in hematopoietic malignancies becomes increasingly compelling (ref. 15 and discussed comprehensively in ref. 7). Conversely, for solid tumors, a particularly appealing idea is that changes in the cellular microenvironment, such as the increasing rigidity of stroma organized around an invasive tumor (26), may directly condition HEF1 activity. The HEF1 paralogue p130Cas has recently been recognized as a mechanosensor hyperphosphorylated in response to stretch (27); HEF1 conserves all the structural elements necessary for a similar response.

Prospect for Therapeutic Exploitation of HEF1

At present, HEF1 is an attractive biomarker of metastatic melanomas. Whether this will be true for other cancers remains to be established; for instance, while HEF1 promotes metastatic behavior in glioblastomas (4), reduced levels of HEF1 transcript characterize an MDA-MB-231 breast cancer cell line selected by serial *in vivo* passages for efficient metastasis to the lung in mice (9). This may represent tumor type–specific HEF1 action. Alternatively, given that the stochiometry of HEF1 and its binding partners is likely to be critical to the signaling function of HEF1, it is possible that these data simply reflect an inhibition of HEF1 function — achieved either by loss of gene product or by altered protein stochiometry resulting from overexpression. Future delineation of these different possibilities will be important for determining HEF1 function in metastatic cancer. No serious attempt has as yet been made to target HEF1 for drug development; as a scaffolding protein with no assigned catalytic function, drug selection strategies are clearly more challenging than for enzymes. However, several points suggest a HEF1-directed targeting strategy is feasible. A peptide aptamer screen has identified discrete peptides that bind and stabilize the HEF1 protein from degradation, implying that it may be possible to identify agents that destabilize the protein (28). Significantly, although HEF1−/− mice manifest some developmental and migration defects, these animals are viable and fertile (15), indicating that loss of HEF1 function can probably be well tolerated in adults. Drugs targeting FAK, Src, BCR-ABL, and TGF-β already in the clinic may be particularly effective in tumors overexpressing HEF1 through limiting HEF1 phosphorylation. Finally, early evidence suggests a potential role for HEF1 in other pathologic conditions, including stroke, rheumatoid arthritis, and human T-cell lymphotrophic virus-1 infection, as well as leukemias and lymphomas (reviewed in refs. 6, 7). A decade after its first description, HEF1 is poised to yield exciting insights into the process of metastasis and may provide an important new target in chemotherapy.

Note added in proof

Ji et al. have now linked upregulation of HEF1 to lung cancer metastasis. Nature 2007;448:807–10.

Acknowledgments

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Molecular basis for HEF1/NEDD9/Cas-L action as a multifunctional coordinator of invasion, apoptosis and cell cycle

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Abstract

Upregulation of the scaffolding protein HEF1, also known as NEDD9 and Cas-L, has recently been identified as a pro-metastatic stimulus in a number of different solid tumors, and has also been strongly associated with pathogenesis of BCR-Abl-dependent tumors. As the evidence mounts for HEF1/NEDD9/Cas-L as a key player in metastatic cancer, it is timely to review the molecular regulation of HEF1/NEDD9/Cas-L. Most of the mortality associated with cancer arises from uncontrolled metastases, thus a better understanding of the properties of proteins specifically associated with promotion of this process may yield insights that improve cancer diagnosis and treatment. In this review, we summarize the extensive literature regarding HEF1/NEDD9/CAS-L expression and function in signaling relevant to cell attachment, migration, invasion; cell cycle; apoptosis; and oncogenic signal transduction. The complex function of HEF1/NEDD9/CAS-L revealed by this analysis leads us to propose a model in which alleviation of cell cycle checkpoints and acquired resistance to apoptosis is permissive for a HEF1/NEDD9/CAS-L-promoted pro-metastatic phenotype.

Keywords

HEF1/NEDD9/CAS-L; NEDD9; Cas-L; metastasis; scaffolding adaptor protein; invasion; mitosis; apoptosis; signal transduction

Introduction

During cancer progression cells undergo multiple genetic and epigenetic changes, promoting increased proliferation potential, resistance to apoptosis, and ability to metastasize. Tumors that remain contained (e.g. carcinomas *in situ*) are generally less malignant, and can be readily treated. Tumors that have progressed to the point of invasiveness and intravasation (entry into blood vessels) usually have acquired multiple lesions that make them refractory to radiation and chemotherapy, and account for much of the morbidity associated with cancer. An important clinical goal is to identify the early genetic or epigenetic changes that cause tumor progression to metastasis.

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A series of studies within the past year have nominated the HEF1/NEDD9/Cas-L protein as an essential switch for pro-metastatic behavior in tumors. HEF1/NEDD9/Cas-L is a component of a small “signature” of genes whose differential expression is associated with metastasizing breast adenocarcinomas (1). HEF1/NEDD9/Cas-L is also a specific effector of the pro-oncogenic FAK kinase, and is required for the migration and invasion of aggressive glioblastomas (2). Most recently, a large “oncogenomics” study used saturation comparative genome hybridization (CGH) mapping in a mouse melanoma metastasis model to identify HEF1/NEDD9/Cas-L as the target of an amplicon associated with melanoma metastasis. The same study then determined that HEF1/NEDD9/Cas-L mRNA and protein expression were elevated in a significant percentage of metastatic melanomas, even in the absence of gene amplification, and that this elevated HEF1/NEDD9/Cas-L expression was required for the metastatic process (3).

In addition, as discussed herein, HEF1/NEDD9/Cas-L has now been shown to have biological actions relevant to immune system maturation; arthritis; stroke; and potentially other clinically important disorders. Over the past decade of study of this protein, a large body of work has accumulated that allows us to begin to assign the molecular action of HEF1/NEDD9/Cas-L relevant to these diseases. Our goal in this review is to describe the complex functions of this protein, discuss the relevance of discrete HEF1/NEDD9/Cas-L functions for disease, and in summary propose strategies for the exploitation of HEF1/NEDD9/Cas-L in therapeutic applications.

Discovery of HEF1/NEDD9/Cas-L

A HEF1/NEDD9/Cas-L partial 3′ untranslated sequence was first described in 1992 by Kumar and coworkers following subtraction cloning to identify cDNAs for genes predominantly expressed in the early embryonic brain. Identified genes were entitled *NEDD* for *Neural precursor cell expressed, Developmentally Down-regulated* (6). A NEDD9 tag was preferentially expressed in embryonic but not adult, brain.

The first two descriptions of the complete HEF1/NEDD9/Cas-L gene and initial functional analysis of the HEF1/NEDD9/Cas-L protein appeared in 1996. As genome resources available in 1996 did not detect homology to the initially described NEDD9 tag, these two reports assigned the gene independent names. Law *et al.* had overexpressed a human cDNA library in *S. cerevisiae* and screened for genes that induced filamentous yeast budding, with the goal of identifying a class of proteins that might coordinately regulate cell polarization and cell cycle in human cells (4). This screen identified a potent yeast cell polarization activity in the carboxy-terminal domains of the HEF1 protein (Human Enhancer of Filamentation 1). Further analysis of the full length HEF1 mRNA and protein in human cell lines revealed HEF1 to be expressed in many cell lines. HEF1 localized to focal adhesions (sites of integrin-dependent attachment to the extracellular matrix) associated with focal adhesion kinase (FAK) and the Abl kinase, and was found to be highly phosphorylated in response to v-Abl transformation (4).

Also in 1996, Minegishi *et al.* identified Cas-L (for Crk-associated substrate-related protein, Lymphocyte type) (5), which is the same gene as HEF1/NEDD9. The goal of the Minegishi study was to clone the gene encoding a protein previously shown to be hyperphosphorylated on tyrosines following ligation of β1-integrins in T cells, and hypothesized to play a role in the process of T cell costimulation. Besides independently establishing the association of Cas-L with FAK, this study also showed Cas-L binding to the integrin effector proteins Crk, Nck, and SHPTP2 following integrin ligation. Together, these two reports focused early interest in HEF1/NEDD9/CAS-L/Cas-L on integrin-dependent signaling pathways in epithelial and lymphoid cells, as discussed below. As of 2007, all three names for the same gene (HEF1, Cas-L, and NEDD9) are in common use.

Protein structure, and definition of the HEF1/NEDD9/CAS-L protein family

Human HEF1/NEDD9/Cas-L is a scaffolding protein, characterized by multiple protein interaction domains. To date, HEF1/NEDD9/Cas-L has no known catalytic activity. In vertebrates, HEF1/NEDD9/Cas-L shares its domain structure and a number of defined protein interactions with two paralogous family members. These paralogs are p130Cas/BCAR1 (7) and EFS/SIN (8–10). Together, the family is often referred to as the "Cas family", for "CRK-associated substrates", based on one of the earliest described protein associations of its members. A single Cas family member exists in *Drosophila* (accession code: CG1212); no strongly homologous Cas protein can be discerned in *C. elegans*, yeasts, or other lower eukaryotes.

Relatively little is known about Efs/Sin, although recent reports suggest important activities in lymphoid cells (11,12). However, in all the following discussions of HEF1/NEDD9/Cas-L, an important consideration is the historical relationship between HEF1/NEDD9/Cas-L and its family member p130Cas. P130Cas was the first member of the family to be discovered, had a clear and immediate relationship to the field of cell adhesion and migration, and is abundant in almost all cell lines and tissues. As a result, many of the initial studies of HEF1/NEDD9/Cas-L in these specific topics were guided by the extensive studies of p130Cas emerging throughout the 1990s (see the excellent reviews by DeFilippi et al, and Bouton et al, (13,14)), on the assumption that because of the considerable sequence homology between the proteins, HEF1/NEDD9/Cas-L would turn out to have similar activities that might be particularly relevant in some tissues. Indeed, while HEF1/NEDD9/Cas-L signaling in adhesion and migration certainly has many common features with p130Cas, the initial assumption that action of these proteins would be near-identical has meant that the exact mechanisms by which HEF1/NEDD9/Cas-L acts in this area have not always been investigated in detail, limiting our the current appreciation of which features of HEF1 and p130Cas are common or unique. For example, unique aspects of HEF1/NEDD9/Cas-L biology relevant to its role in metastasis have emerged only recently (enumerated below).

Figure 1 displays the most notable structural features of the human 834 amino acid HEF1/NEDD9/Cas-L protein. Amino acids ~10–65 encode an SH3 domain, which confers interactions with partner proteins bearing a poly-proline motif (15). Amino acids ~90–350 are characterized by a large number of SH2 binding sites: tyrosine-containing motifs that when phosphorylated allow binding to partner proteins bearing SH2 domains (16). This part of the protein is often termed the "substrate domain". Amino acids 350–650 are characterized by a very large number of serine residues. Studies of p130Cas have identified a four-helix bundle encompassed within this region that may serve as a protein-binding site, and is conserved structurally among other focal adhesion-associated proteins (17). Computational modeling of the HEF1/NEDD9/Cas-L amino acid sequence using techniques to identify conserved folds (18) suggests this four-helix bundle exists in HEF1/NEDD9/Cas-L (unpublished results). Finally, the carboxy-terminal domain of HEF1/NEDD9/Cas-L is highly conserved among Cas proteins, although its structure has not yet been solved. Some studies have shown that this domain selectively interacts with a subset of helix-loop-helix (HLH) proteins, allows homo- and hetero-dimerization of Cas proteins, and has suggestive sequence motifs that indicate it may contain an embedded HLH sequence (4,19); the latter point has not been rigorously demonstrated.

Although part of the HEF1/NEDD9/Cas-L protein pool is cytoplasmic, in response to intrinsic and extrinsic cues HEF1/NEDD9/Cas-L concentrates at focal adhesions, and at the centrosome and mitotic spindle (4,20,21). Focal adhesion targeting sequences for HEF1/NEDD9/Cas-L and other Cas proteins are found in the SH3-domain and the conserved carboxy-terminal domain (22,23). Some recent studies of p130Cas have suggested that the substrate domain is

intrinsically unstructured, but that physical stretching based on forces generated through the N- and C-terminal attachments at focal adhesions exposes the embedded SH2-binding sites for phosphorylation and binding. Given that very similar structural features are found in HEF1/NEDD9/Cas-L and Efs, this raises the possibility that the entire Cas family of proteins act at least in part as mechanosensors (24–26). As discussed below, some biological stimuli associated with apoptosis result in the caspase-mediated cleavage of HEF1/NEDD9/Cas-L into stable processed products, which function as dominant negatives for the action of the full-length protein.

Sequences targeting HEF1/NEDD9/Cas-L to the centrosome are concentrated in the substrate-binding domain (21). Both N-terminal and C-terminal sequences of HEF1/NEDD9/Cas-L confer interaction with SMAD3 and the proteasomal machinery, which allows rapid turnover of HEF1/NEDD9/Cas-L in response to diverse biological signals (27,28). Importantly, these latter interactions with the centrosome and proteasome so far appear to be unique to HEF1/NEDD9/Cas-L among the Cas proteins. Finally, it is worth noting that p130Cas and Efs contain poly-proline domains that interact with SH3-domains in partner molecules, but these poly-proline stretches are absent from HEF1/NEDD9/Cas-L.

Regulation of HEF1/NEDD9/Cas-L

Over the past decade, HEF1/NEDD9/Cas-L has emerged as a protein subject to dynamic and complex regulation, which in turn can influence multiple different biological processes. This section will focus primarily on the regulation of HEF1/NEDD9/Cas-L at the levels of transcription and post-translational modification in adherent (predominantly epithelial) cell lines, although briefly summarizing key data from lymphoid and myeloid cells that is discussed in greater detail in later sections. The emphasis here is to define the cellular context in which HEF1/NEDD9/Cas-L is normally abundant and active, versus minimally expressed or inactive, and to establish the cellular machinery responsible for conversion between these states.

Phosphorylation control of HEF1/NEDD9/Cas-L

HEF1/NEDD9/Cas-L is commonly visualized from SDS-PAGE gels as both a 105 and a 115 kDa protein (29), significantly higher than its predicted molecular weight of ~93 kDa, and reflecting the extensive phosphorylation of HEF1/NEDD9/Cas-L. Such abundant phosphorylation is a common feature of Cas family proteins: indeed, p130Cas was first targeted for study based on its dramatic increase in phosphorylation arising following oncogenic transformation of cells, or cell attachment to integrins (e.g. (30)). As phosphorylation control is a critical component of HEF1/NEDD9/Cas-L regulation, it is discussed first.

From its first identification (4,5), FAK and Src family kinases were implicated as important regulators of HEF1/NEDD9/Cas-L phosphorylation. In a simple model for HEF1/NEDD9/Cas-L action (see Figure 2), initial ligation of integrins during cell attachment activates the integrin-associated FAK (or its paralog, RAFTK/CAK-β/Pyk2), which phosphorylates HEF1/NEDD9/Cas-L on a tyrosine near the carboxy-terminus. This phospho-tyrosine creates a binding site for Src (or its paralogs Fyn, Lyn, and others), which extensively tyrosine-phosphorylates HEF1/NEDD9/Cas-L in the substrate domain: this “activation” process enables HEF1/NEDD9/Cas-L to bind downstream effectors that promote migration, invasion, and proliferation-related signaling. Many groups have investigated this core regulation process, in multiple cell types (e.g. (31,32)). Relevant to cancers, overexpression or constitutive activation of FAK and Src can override the need for integrin ligation to phosphorylate HEF1/NEDD9/Cas-L. Interestingly, studies of the HEF1/NEDD9/Cas-L-related proteins Efs/Sin and p130Cas have shown that these proteins reciprocally activate Src (8,33,34), raising the possibility that a similar activity exists for HEF1/NEDD9/Cas-L. However, as the C-terminal poly-proline domain of p130Cas and Efs plays a role in the activation of Src (35), and HEF1/NEDD9/Cas-

L lacks these sequences, thus HEF1/NEDD9/Cas-L may not have identical Src-stimulating activity to the other family members. Separately, some studies have implied that FAK is a HEF1/NEDD9/Cas-L effector, as well as activator (3). Hence, if the interaction between the three proteins is mutually activating, the idea of “upstream” and “downstream” factors may be misleading, particularly under the conditions of significantly overexpressed HEF1/NEDD9/Cas-L observed in metastasis (1,3).

Cell adhesion also regulates the conversion of p105 to p115 HEF1/NEDD9/Cas-L, as demonstrated by the disappearance of p115 in suspended cells and a corresponding increase in p105, and the appearance of p115 and reduction of p105 HEF1/NEDD9/Cas-L upon re-adhesion (28,31,36). Both p105 and p115 HEF1/NEDD9/Cas-L are tyrosine phosphorylated, and p115HEF1/NEDD9/Cas-L is hyper-phosphorylated in relation to p105 HEF1/NEDD9/Cas-L (29). However, contrary to initial expectation, p105 HEF1/NEDD9/Cas-L contains more phospho-tyrosine than the p115 species (28,31), indicating that the readily detected mobility shift does not directly reflect HEF1/NEDD9/Cas-L tyrosine phosphorylation status. Rather, the 115kDa form of HEF1/NEDD9/Cas-L has been shown in some cell types to be predominantly a serine/threonine phosphorylation modification of p105 HEF1/NEDD9/Cas-L (31). With the notable exception of Aurora A kinase, discussed below in the section on cell cycle, little is known about the identity of kinases phosphorylating HEF1/NEDD9/Cas-L on serines and threonines during the adhesion process, or the specific phosphorylation events leading to the p105/p115 conversion.

The phosphorylation status of HEF1/NEDD9/Cas-L is influenced by the integrity of the actin cytoskeleton (28,31). Actin filament disrupting drugs induce the dephosphorylation of p115 HEF1/NEDD9/Cas-L, inducing PP2A serine/threonine protein-phosphatases, which convert p115 to p105 HEF1/NEDD9/Cas-L (36). This action is specific to the actin cytoskeleton as disruption of microtubule and intermediate filament networks do not influence the p105/p115 ratio (36). Bargon and coworkers also demonstrated the loss of p115 kDa HEF1/NEDD9/Cas-L upon inhibition of Rho kinase, an important regulator of actin cytoskeleton rigidity (37). Further, cells grown in serum free media rapidly lose the p115kDa isoform of HEF1/NEDD9/Cas-L whereas the p105 is largely unaffected: in cells that were allowed to establish focal adhesions prior to serum starvation, the p115kDa HEF1/NEDD9/Cas-L is maintained (23). These results suggest that the significant physical rearrangements that occur during formation of focal contacts (38), and potentially the ability to generate actin-based pulling forces that might activate a mechanosensor (26), integrally regulate HEF1/NEDD9/Cas-L phosphorylation. Extending this line of speculation, it is now appreciated that changes in tumor microenvironment occurring during tumor progression prior to metastasis result in striking changes in the rigidity of the extracellular matrix that binds and activates integrins (39): the role of such changes in influencing actin cytoskeletal dynamics, and hence HEF1/NEDD9/Cas-L, remain unexplored.

HEF1/NEDD9/Cas-L phosphorylation is not only responsive to adhesion. For example, stimulation of the G protein-coupled receptor (GPCR) for calcitonin also induces tyrosine phosphorylation of HEF1/NEDD9/Cas-L, dependent on both cell adhesion and the integrity of the actin cytoskeleton (40). TGF-β1 treatment induces substantial tyrosine phosphorylation of HEF1/NEDD9/Cas-L; interestingly, this is independent of cell adhesion and an intact actin cytoskeleton (31). PDGF stimulation of glioblastoma cells specificallly increases the phosphorylation of HEF1/NEDD9/Cas-L; despite the presence of abundant p130Cas in the same cells, only experimentally downregulated HEF1/NEDD9/Cas-L inhibited basal and PDGF-stimulated migration (2). In a neuroblastoma cell line, stimulation of the muscarinic receptor induces tyrosine phosphorylation of HEF1/NEDD9/Cas-L; this phosphorylation is inhibited by oxidative stress (41). HEF1/NEDD9/Cas-L is also tyrosine phosphorylated in neurons of the cerebral cortex and hippocampus following global ischema in adult rat brains

(42). The roles of HEF1/NEDD9/Cas-L in these additional pathways remain only minimally explored to date. Finally, accumulation of serine/threonine-phosphorylated p115 HEF1/NEDD9/Cas-L marks the G2 phase of cell cycle; the important role of this form of HEF1/NEDD9/Cas-L is discussed below.

Transcriptional control of HEF1/NEDD9/Cas-L

In quiescent cultured cells, the levels of endogenous HEF1/NEDD9/Cas-L are very low, but levels rise rapidly as cells are induced to cycle (29). Although study of transcriptional induction of HEF1/NEDD9/Cas-L has been underexplored, some inducing factors have been identified. TGF-β treatment induces HEF1/NEDD9/Cas-L protein expression in part through the induction of HEF1/NEDD9/Cas-L mRNA transcription (31). The vitamin A metabolite all-trans retinoic acid (asRA) induces HEF1/NEDD9/Cas-L transcription in two different neuroblastoma cell lines, suggesting the involvement of HEF1/NEDD9/Cas-L in neural development (43,44): A putative retinoic acid response element in the 5′ region of the NEDD9 promoter specifically binds a RXR/RAR heterodimer and forms a higher molecular weight complex upon addition of a retinoic acid receptor specific antibody (43). HEF1/NEDD9/Cas-L mRNA is up-regulated in response to progesterone receptor A overexpression (45), but has been reported to be down-regulated in ER-alpha transfected osteosarcoma cells (46) and in estrogen-treated MCF-7 cells (47). HEF1/NEDD9/Cas-L is transcriptionally upregulated in neurons of the cerebral cortex and hippocampus following global ischema in adult rat brains (42), although the signaling pathways responsible are not known.

It is likely that stimulated HEF1/NEDD9/Cas-L transcription will turn out to be important during cancer metastasis. High throughput studies have shown HEF1/NEDD9/Cas-L mRNA is upregulated in ovarian cancer versus normal ovarian epithelium (48), and in a high percentage of metastatic melanomas lacking evidence of amplification of the HEF1/NEDD9/Cas-L gene (3). This upregulation may in part reflect the association of elevated HEF1/NEDD9/Cas-L expression with actively cycling cells (29); alternatively, activation of specific signaling pathways may also play a role. Intriguingly, a genome-scale location analysis of binding sites for transcription factors associated with stem cells has recently shown that SOX2 and NANOG co-occupy the HEF1/NEDD9/Cas-L promoter (49); the relevance of this observation to cancer has not yet been explored.

Proteolysis of HEF1/NEDD9/CAS-L

The HEF1/NEDD9/Cas-L protein is regulated by controlled protein cleavage and degradation and this control is relevant to several different biological systems. First, in actively cycling cells, HEF1/NEDD9/Cas-L is proteasomally degraded at the end of mitosis, leading to very low levels of the protein detected in early G1 (21,29). Second, during anoikis/apoptosis, HEF1/NEDD9/Cas-L is cleaved at specific DLVD and DDYD caspase cleavage sites, resulting in the replacement of HEF1/NEDD9/Cas-L with shorter processed products that have the capacity to act as dominant negatives for HEF1/NEDD9/Cas-L -related signaling (20,23): the functional consequences of this process are discussed at length in the section on apoptosis, below. We note that although similar cleavages were suggested as occurring in mitotic cells (29), it now appears that this processing is primarily limited to apoptotic cells.

Besides regulating HEF1/NEDD9/Cas-L at the transcriptional level, TGF-β also regulates HEF1/NEDD9/Cas-L at the level of proteolysis. Several groups have demonstrated direct interaction of HEF1/NEDD9/Cas-L with SMAD proteins, ubiquitin ligases and associated factors that induce proteolytic cleavage and degradation of target proteins in response to TGF-β signaling (27,28,50–52). These interactions can cause proteolysis of HEF1/NEDD9/Cas-L (28). Reciprocally, HEF1/NEDD9/Cas-L can modulate the activity of the SMAD proteins, limiting TGF-β signaling output (51). This intimate connection between TGF-β and HEF1/

NEDD9/Cas-L may prove to be important for the action of HEF1/NEDD9/Cas-L in metastasis. Notably, TGF-β signaling converts from growth-inhibitory to growth-promoting during tumor cell progression (53). Related to this, TGF-β and Ras pathways collaborate to induce invasive and metastatic behavior; HEF1/NEDD9/Cas-L promotion of metastasis has been suggested to depend on Ras pathway activation (3). The possibility that HEF1/NEDD9/Cas-L may act as a critical modulator or surrogate for TGF-β in metastasis remains to be explored.

Pro- and anti-metastatic activities of HEF1/NEDD9/Cas-L: a balance of forces

HEF1/NEDD9/Cas-L plays important roles in the regulation of at least three distinct classes of biological process. These include 1) attachment, migration and invasion; 2) apoptosis; and 3) cell cycle. To date, HEF1/NEDD9/Cas-L levels have been shown as being sharply elevated in metastasis (1,3). In cultured cells, elevation of HEF1/NEDD9/Cas-L protein commonly promotes migration and invasion, but can also simultaneously induce apoptosis and mitotic defects that trigger cell cycle arrest checkpoints. Based on the data summarized below, we propose that an important reason HEF1/NEDD9/Cas-L is upregulated at the point of metastasis, rather than earlier in cancer development, is that cells must acquire prior genetic lesions that counteract HEF1/NEDD9/Cas-L-dependent cell death and/or arrest: otherwise, cells are unable to tolerate increased expression of HEF1/NEDD9/Cas-L.

HEF1/NEDD9/CAS-L positively regulates attachment, migration, and invasion

The process of cell migration requires complex coordination between the cell polarity machinery, the actin and microtubule cytoskeletons, membrane dynamics and focal adhesion turnover. Through their directed formation and break down, focal adhesions provide changing points of force concentration against the surrounding extracellular matrix, while the actin cytoskeleton provides the contractile force necessary for cellular movement (reviewed in (54)): HEF1/NEDD9/Cas-L, residing at focal adhesions, is positioned to interact with many of the key proteins coordinating migration. Notably, cell migration is critical during many non-pathological cellular processes (for example, embryogenesis and inflammation), and is aberrantly activated in many cancers. It is likely that the basic cell migration machinery that is used during non-pathological cell movement is co-opted during the development of metastatic cancer, either by the de-regulation of inhibitory molecules or stimulatory molecules. Indeed, recent emerging data suggests that changes in HEF1/NEDD9/Cas-L expression are important in non-pathological movement of hematopoietic lineage cells (55), and also play a vital role in the development of metastatic capability in glioblastoma (2), melanoma (3) and breast cancer (1).

HEF1/NEDD9/Cas-L acts at a number of key points in the cell migration pathway (Figure 3). Cells overexpressing HEF1/NEDD9/Cas-L spread more on 2-dimensional supports (56), while overexpression of a carboxy-terminal peptide of HEF1/NEDD9/Cas-L comparable to a naturally occurring caspase cleavage product (p28) promotes cell rounding and loss of focal adhesions (23). These results suggest that like p130Cas (57), HEF1/NEDD9/Cas-L directly regulates the dynamics of focal adhesion formation and disassembly. p130Cas requires an intact substrate-binding domain to induce the formation of polymerized actin during migration (58), and it is likely that HEF1/NEDD9/Cas-L has a similar structural requirement, although this has not been experimentally tested.

HEF1/NEDD9/Cas-L interaction with FAK has been demonstrated to be a key initiating event both in *in vitro* assays of migration (2,3,59–61) and *in vivo* invasion (3). Following phosphorylation by Src and FAK, HEF1/NEDD9/Cas-L interacts with the adaptor molecule Crk (5); the interaction of Crk with p130Cas has similarly been shown to be a key event in p130Cas promotion of cell migration (62). Crk association with p130Cas subsequently recruits the exchange factor DOCK180, resulting in the activation of the GTPase Rac, which feeds into

a well-described pathway involving the stimulation of membrane ruffling and extension via the activation of the Arp2/3 actin polymerization complex (63,64) and kinases such as Pak (65). Crk also recruits C3G, thereby activating a second promigratory pathway proceeding through a second GTPase, Rap1 (24). Currently, these pathways have not been extensively mapped downstream of HEF1/NEDD9/Cas-L, although it is likely that HEF1/NEDD9/Cas-L and p130Cas act similarly. Notably, the substrate domain of HEF1/NEDD9/Cas-L which contains the canonical Crk binding sites (66) is required for HEF1/NEDD9/Cas-L promotion of cell migration (56,59), but whether this correlates with enhanced activation of Rac and Rap has not been directly investigated.

Recently, it has been proposed that interaction of HEF1/NEDD9/Cas-L with distinct signaling pathways might separately promote cell migration and invasion. These include the interaction of HEF1/NEDD9/Cas-L with the Cas family-binding proteins variously known as BCAR3/AND-34/SHEP2/Nsp2, and CHAT-H/SHEP1. These paralogous molecules have been proposed to regulate the activity of a number of GTPases (67–72) and hence also result in the activation of downstream effectors such as PAK. The relative use of Crk- versus BCAR3-dependent signaling pathways is likely to vary in a cell type-specific manner, and dependent on the specific upstream initiating stimuli; this area has not been well investigated.

In vitro studies have demonstrated that the overexpression of HEF1/NEDD9/Cas-L can promote cell migration in a variety of cell types and results in both an enhanced velocity of random cell migration (56) and enhanced haptotactic response (56,59–61), while HEF1/NEDD9/Cas-L knock-down impairs chemotaxis (55). Importantly, it appears that HEF1/NEDD9/Cas-L and p130Cas may have tissue-specific effects on cell motility. In a key finding, Natarajan and co-workers demonstrated that HEF1/NEDD9/Cas-L, but not p130Cas, promotes motility and invasion in glioblastoma cells (2). In a related finding, the perturbation of lymphocyte trafficking observed in the HEF1/NEDD9/Cas-L knockout mouse model suggests that p130Cas can not compensate for HEF1/NEDD9/Cas-L migratory function at least in this particular tissue (55). Notably, Rho kinase inhibition (37), and FAK knock-down or dominant negative inhibition (3,59) are so far the only pathways demonstrated to reverse migration promoted by experimentally induced HEF1/NEDD9/Cas-L expression. Interestingly, HEF1/NEDD9/Cas-L can promote neurite-like extensions in epithelial cells following the inhibition of Rho kinase (37), suggesting the existence of distinct, downstream cell polarization effectors. The processes of cell migration and neurite extension have many intriguing parallels (73,74), and both require co-ordination of cell morphology with adhesion dynamics.

Moving further downstream, both p38 mitogen activated protein kinase (MAPK) and Extracellular Related Kinase 1/2 (Erk1/2) have been shown to be activated downstream of HEF1/NEDD9/Cas-L induced expression, yet neither of these pathways appeared to account for HEF1/NEDD9/Cas-L promoted cell migration (56). Jun-N-terminal kinase (JNK) is also activated downstream of induced HEF1/NEDD9/Cas-L (20); thus, JNK activation has been implicated in HEF1/NEDD9/Cas-L promoted cell migration, as has been shown for p130Cas (75), however, a requirement for JNK has not been directly experimentally tested. A number of genes have been identified that are transcriptionally activated downstream of HEF1/NEDD9/Cas-L overexpression, that are likely players in cell migration and invasion (56). The induced molecules included multiple matrix metalloproteinases (MMPs), disintegrin, myosin light chain kinase (MLCK), Rho kinase, Nck interacting kinase (Nik), components of ephrin signalling pathways, extra-cellular matrix components, transforming growth factor receptors and the ErbB2/Her2/Neu receptor (56). Currently, the functional role of induction of these genes by HEF1/NEDD9/Cas-L overexpression has not been extensively investigated, and it will be interesting to see the extent to which any of these molecules represent HEF1/NEDD9/Cas-L-pathway-specific targets. However, the role of proteins such as MMPs, disintegrin, and some ephrins in promoting invasion and metastasis is evident from the current literature.

HEF1/NEDD9/CAS-L and apoptosis

The orderly process of apoptosis is coordinated via the activation of caspases, enzymes that cleave proteins. Cells undergoing apoptosis exhibit characteristic morphological features of cell rounding and membrane blebbing and accompanying this morphology is the disassembly of focal adhesions. Caspase cleavage of proteins during apoptosis can either activate the targeted molecule (76), while in other cases cleavage may disable the molecule (77). Whether the molecule is activated or disabled, the unifying feature is that the cleavage of these molecules appears to be necessary for apoptosis to occur.

Both HEF1/NEDD9/Cas-L (77) and p130Cas (78) are targeted for cleavage by caspases (Figure 4). The cleavage of HEF1/NEDD9/Cas-L can be inhibited by integrin receptor activation, suggesting that HEF1/NEDD9/Cas-L may act as a type of sensor at focal adhesion sites (23). Correspondingly, unligated integrin may be sufficient to cause the activation of caspases (79). As with FAK and gelsolin (76,77), overexpression of a HEF1/NEDD9/Cas-L C-terminal 28 kDa peptide equivalent to the naturally produced caspase-derived peptide stimulated apoptosis in MCF-7 breast cancer cells (20), and in a number of other cell types (unpublished results). Importantly, overexpression of full length HEF1/NEDD9/Cas-L in some cell types also eventually triggers apoptosis. One idea is that low-level cleavage of overexpressed HEF1/NEDD9/Cas-L produces a small amount of p28, which in turn induces disassembly of focal adhesions and activates anoikis (detachment-initiated cell death, (80)). Of note, HEF1/NEDD9/Cas-L overexpression in MCF-7 cells initially stimulates cell migration (56) but this eventually culminates in the promotion of apoptosis (20), suggesting a change in protein behavior over time. An alternative interpretation is that the apoptosis arises subsequent to activation of mitotic checkpoints, as discussed below.

However, the overexpression of HEF1/NEDD9/Cas-L does not universally induce apoptosis. HEF1/NEDD9/Cas-L overexpression in the apparent absence of apoptosis has been demonstrated in Jurkat cells (59), glioblastoma (2), melanoma (3) and ALT (61). Notably, each of these models represent highly metastatic and invasive cells; in contrast the MCF-7 breast cancer cells are only weakly tumorigenic in nude mice (81). The conversion to a metastatic phenotype depends on corresponding activation of cell survival pathways; thus HEF1/NEDD9/Cas-L expression may collaborate to promote cell migration only in the context of enabling survival pathway activation.

HEF1/NEDD9/Cas-L and cell cycle control

One of the most unexpected aspects of HEF1/NEDD9/Cas-L biology has emerged within the past two years. It had been appreciated since 1998 that the abundance of the HEF1/NEDD9/Cas-L protein was strongly regulated by cell cycle, with very low levels of the protein detectable in quiescent or G1 populations, increasing levels during S phase, and peak abundance in late G2/M (29). Parallel immunofluorescence analysis suggested that HEF1/NEDD9/Cas-L might associate with the mitotic spindle, but the specificity of the observed staining pattern and the biological role for HEF1/NEDD9/Cas-L in mitosis (if any) remained to be determined.

In 2005, Pugacheva and Golemis used multiple approaches to show that HEF1/NEDD9/Cas-L concentrates at the centrosome in G2 phase, then at mitotic entry moving along the mitotic spindle to the mitotic midzone, and finally localizing at the midbody at cytokinesis. Importantly, cells overexpressing HEF1/NEDD9/Cas-L accumulate multipolar spindles and supernumerary centrosomes, with these defects arising from defective cytokinesis (21,82). Conversely, cells with depleted HEF1/NEDD9/Cas-L have prematurely separated centrosomes in interphase that demonstrated hallmarks of immature maturation, and are deficient in microtubule organizing activity at mitosis, leading to an abundance of monopolar or

asymmetric spindles (21). These cells also have difficulty in completing mitosis, and commonly experience cleavage furrow regression and accumulation of binucleate cells (82). Cells passing through mitosis with aberrant HEF1/NEDD9/Cas-L levels commonly arrest in G1 phase of cell cycle, compatible with triggering of mitotic checkpoints, and ultimately are cleared by apoptosis (83).

Although the complete definition of HEF1/NEDD9/Cas-L activities in cell cycle progression requires much more work, several mechanisms are already clear (Figure 5). Prior to mitotic entry, HEF1/NEDD9/Cas-L functionally interacts with a centrosomal kinase, Nek2. Nek2 activation in G2 leads to separation of centrosomes; in the absence of HEF1/NEDD9/Cas-L, Nek2 is prematurely activated (21), potentially explaining the premature centrosomal splitting seen in HEF1/NEDD9/Cas-L-depleted cells. Second, HEF1/NEDD9/Cas-L directly associates with the Aurora A kinase at the centrosome at the G2/M transition, and is required for Aurora A activation. Timed activation of Aurora A is essential for orderly progression through mitosis, and defects in Aurora A signaling yield phenotypes similar to those observed with defective HEF1/NEDD9/Cas-L (discussed in (83)). Third, HEF1/NEDD9/Cas-L interacts with ECT2 (*Drosophila* Pebble), a RhoA GDP-GTP exchange factor (GEF) that specifically activates RhoA in mitosis. Timed and spatially controlled activation of RhoA governs multiple stages of mitosis, through regulating cortical actin contractility (84–86). Cells with elevated HEF1/NEDD9/Cas-L expression experience abnormally enhanced RhoA activity, which contributes to the arrest of these cells at the point of abscission (82).

There are other candidate signaling partners for HEF1/NEDD9/Cas-L in regulation of mitosis. As one example, HEF1/NEDD9/Cas-L interacts with the LIM domain protein zyxin (87); zyxin has been shown to interact with the tumor suppressor LATS1 at the mitotic spindle to time mitotic entry (88). As another example, the Src kinase, an important HEF1/NEDD9/Cas-L partner in interphase cells, has long been known to be hyperactivated in mitosis, and to contribute to mitotic spindle assembly and changes in adhesion (89,90); a potential role with HEF1/NEDD9/Cas-L would not be unreasonable. Levels of HEF1/NEDD9/Cas-L drop sharply at the end of mitosis as a result of proteasomal degradation (29). Whether the factors targeting HEF1/NEDD9/Cas-L to the proteasome are the same now being defined in interphase cells, or are completely independent, is another interesting question.

Biological Activities of HEF1/NEDD9/CAS-L in lymphoid and myeloid cells

Until the recent expansion into the realm of cell cycle, most of the HEF1/NEDD9/Cas-L studies performed in adherent cells focused on integrin ligation and attachment signaling pathways relevant to cancer. In contrast, while studies of HEF1/NEDD9/Cas-L in the hematopoietic system have identified similar signaling in relation to integrin and cancer, this work has also addressed the biology of HEF1/NEDD9/Cas-L in processes as diverse as T-cell receptor signaling, autoimmunity, and response to viral infection. These studies of HEF1/NEDD9/Cas-L in the immune system provide a useful complement to consideration of HEF1/NEDD9/Cas-L function in adherent cell types; they are discussed in greater depth in a recent review by Seo and colleagues (91).

Integrin-dependent signaling

The first identification of HEF1/NEDD9/Cas-L in lymphoid cells observed that extensive tyrosine phosphorylation of the protein following integrin crosslinking led to association of the protein with adaptor proteins including Crk, Nck, and the phosphatase SHPTP2 (5). A number of subsequent studies delineated in detail the role of HEF1/NEDD9/Cas-L in integrin-dependent signaling in T cells (59,60,92–95), B cells (96–99), and myeloid cells (100). The general outline of HEF1/NEDD9/Cas-L signaling interactions during response to integrins essentially parallels that observed in adherent cells. Following ligation of integrins, FAK or

the lymphocyte-associated FAK paralog RAFTK/CAK-β/Pyk2 binds and phosphorylates HEF1/NEDD9/Cas-L (96). This is followed by HEF1/NEDD9/Cas-L association with a Src family kinase: this may be Fyn, Lyn, or Lck rather than Src (98,101). Downstream effectors binding HEF1/NEDD9/Cas-L following this phosphorylation include Crk-l, leading to recruitment of C3G and activation of Rap1 and other pro-motility proteins (e.g. (100)). Indeed, HEF1/NEDD9/Cas-L is required for integrin-dependent migration of lymphoid cells, and overexpression of HEF1/NEDD9/Cas-L can induce such migration, again paralleling adherent cells (59,60).

Co-stimulation and response to non-integrin surface receptors

Beyond its role in integrin-dependent signaling, HEF1/NEDD9/Cas-L has an important additional role in lymphoid and myeloid cells. Proliferation and differentiation of lymphoid cells depends on integrated signaling between integrins and either the B-cell receptor/antigen receptor, for B cells, or the T cell receptor (TCR)/CD3, for T cells. A number of studies have shown that HEF1/NEDD9/Cas-L is phosphorylated following antigen receptor/TCR stimulation independent of integrin ligation, and is an important coordinator of co-stimulation (60,94,97,98,102,103). Structure-function experiments indicate that there are some differences between the utilization of HEF1/NEDD9/Cas-L in response to integrin versus TCR or antigen receptor ligation: for example, the FAK-binding SH3 domain of HEF1/NEDD9/Cas-L is required for HEF1/NEDD9/Cas-L phosphorylation following integrin ligation, but not for HEF1/NEDD9/Cas-L phosphorylation following TCR activation (103). HEF1/NEDD9/Cas-L also is activated and plays a role in cell response following ligation of the tetraspanin CD82, in T cells (104), and of the Fc receptor, in U937IF cells (100).

These and further integrating activities may involve some additional HEF1/NEDD9/Cas-L partners. For example, CHAT-H is a<u>h</u>ematopoietically expressed paralog of the ubiquitously expressed CHAT (<u>C</u>as/<u>H</u>EF-<u>a</u>ssociated signal<u>t</u>ransducer, also known as Nsp3/Shep-1, (105)) (69,70,106). CHAT and CHAT-H are adaptor proteins, containing SH2 domains, and C-terminal association domains that mediate association with HEF1/NEDD9/Cas-L, and have properties of GEF domains (71); however, CHAT-H has a unique N-terminal extension. Importantly, CHAT-H has now been shown to signal through Rap1 to coordinate chemokine and integrin-dependent cell migration responses in T cells (72). AND-34 (also known as Nsp2 and BCAR3, (105)) is structurally related to CHAT-H. AND-34 also associates with HEF1/NEDD9/Cas-L in B cells to activate downstream signaling cascades relevant to cell migration (65,67,107). As discussed above, AND-34 also modulates cellular responsiveness to estrogens, and when overexpressed can cause estrogen resistance. Estrogens are now appreciated as exerting profound influence on immune function (108): the possible role of an interaction between HEF1/NEDD9/Cas-L and AND-34 in these responses has not been explored.

BCR-Abl

In the first report of HEF1/NEDD9/Cas-L, overexpressed v-Abl bound to- and induced-tyrosine phosphorylation of HEF1/NEDD9/Cas-L (4). Although the significance of the HEF1/NEDD9/Cas-L -Abl interaction in epithelial or other non-hematopoietic cell lineages remains to be explored, HEF1/NEDD9/Cas-L may be an important player in the pathogenesis of leukemias dependent on the BCR-Abl (Philadelphia chromosome) translocation product (109–112). HEF1/NEDD9/Cas-L is extensively tyrosine phosphorylated in BCR-Abl-transformed cells, promoting interaction with Crk-L; Crk-L is a major *in vivo* binding partner and substrate of the deregulated BCR-Abl tyrosine kinase and functions as a molecular link with other signaling proteins. Hyperactivation of HEF1/NEDD9/Cas-L -Crk-L coupling by BCR-Abl might explain the enhanced adhesion properties of leukemic cells in the bone marrow. In mouse models, Crk-L co-overexpression with BCR-ABL promotes leukemogenesis, but deletion of CRK-L is unable to block leukemogenesis (113,114); hence,

HEF1/NEDD9/Cas-L signaling to Crk-L may not be the only important BCR-ABL effector pathway, although more work is required to firmly establish this point. To date, the role of other HEF1/NEDD9/Cas-L partners in BCR-ABL-dependent leukemias have not been explored. We speculate that given the frequent co-activation of Ras-dependent signaling in BCR-ABL leukemias (115), and in light of the recently established close connection between HEF1/NEDD9/Cas-L and Ras in metastasis (3), the interaction of HEF1/NEDD9/Cas-L with proteins involved in Ras signaling may be a particularly fertile area for further exploration.

Inflammatory response, viral pathogenesis, and immune system maturation

In the past several years, a number of groups have begun to explore the role of HEF1/NEDD9/Cas-L in pathogenic conditions that specifically involve the immune system. Among these efforts, some particularly interesting findings have been made in the areas of HTLV-1 infection, rheumatoid arthritis, and differentiation of B cells (55,61,116). Adult T-cell lymphoma (ALT) can be induced by the human T-cell leukemia virus 1 (HTLV1) (117). The transforming agent of HTLV1 is the Tax protein and transgenic Tax expression can independently induce spontaneously metastasizing leukemias (118), and can also induce a syndrome of rheumatoid arthritis (RA) (119) similar to arthritis syndromes found in human patients with HTLV-1 infections (120). HEF1/NEDD9/Cas-L binds directly to Tax, and overexpression of HEF1/NEDD9/Cas-L limits the ability of Tax to activate NF-kB (61), thus inhibiting Tax-induced transcription of cell proliferation genes (117). Importantly, the migration of splenocytes from Tax transgenic mice with arthritis (ATg) is much higher than that of Tax transgenic mice without arthritis (NTg). HEF1/NEDD9/CAS-L expression and tyrosine phosphorylation are increased in ATg mice and this is accompanied by enhanced autophosphorylation of the Src family kinases Fyn and Lck. Immunohistochemical analysis also demonstrated a large number of HEF1/NEDD9/Cas-L-positive lymphocytes migrating into the affected joints of Atg mice. Furthermore, in human RA, HEF1/NEDD9/Cas-L-positive lymphocytes infiltrate the inflammatory lesions (61,116). Together, these findings raise the possibility that HEF1/NEDD9/Cas-L expression levels can feedback to determine NF-kB activation; more investigation is required.

With their creation of a HEF1/NEDD9/Cas-L knockout mouse, Seo and co-workers recently provided an invaluable tool for the physiological study of HEF1/NEDD9/Cas-L (55). Although much characterization remains to be done, initial analysis focused on the immune system maturation of these animals reveals multiple defects affecting marginal zone B cells and population of secondary lymphoid organs. These deficiencies appear to involve changes in cell adhesion, migration, and chemotactic response (55), as predicted by the cell-based studies described above. While all of these phenotypes are interesting, the striking loss of chemokine responsiveness observed supports the idea that HEF1/NEDD9/Cas-L may act as a component of additional GPCR signaling pathways beyond calcitonin, discussed above. These animals should be an invaluable tool in further exploring HEF1/NEDD9/Cas-L functions in pathological conditions of the immune system and other organs, as discussed further below.

HEF1/NEDD9/CAS-L in Development

The role of HEF1/NEDD9/Cas-L in the development and non-cancerous cell signaling of normal mammalian tissues is only beginning to be addressed. The HEF1/NEDD9/Cas-L knockout mouse created by Seo *et al.* is viable and fertile as a homozygote, and does not display gross abnormalities in any tissues (55). This is in marked contrast to the p130cas knockout, which is embryonic lethal at day 11 (121), and suggests that at least some critical HEF1/NEDD9/Cas-L functions can be fully compensated for by p130Cas or other proteins. Besides the delineation of immune system defects described above, a detailed analysis of the HEF1/NEDD9/Cas-L knockout animals is in progress, and it is likely that more defects will be found. Consideration of data produced by a number of studies that have been performed to date

suggests specific developmental processes that are likely to prove fertile in yielding a role for HEF1/NEDD9/Cas-L and they are discussed below.

Several distinct studies suggest that HEF1/NEDD9/Cas-L will be important for appropriate neuronal differentiation and brain development. In 1992, it was noted that the HEF1/NEDD9/Cas-L transcript is abundant in early murine brains, but downregulated by approximately day 10 of embryonal development (6). The first substantial experimental evidence for HEF1/NEDD9/Cas-L in brain development was provided by Merrill and co-authors in 2004 (43, 44), who used a subtractive cDNA library prepared from the human neuroblastoma cell line, SH-SY5Y to identify genes induced by the vitamin A metabolite, All-*trans* retinoic acid (atRA), an established regulator of brain development. HEF1/NEDD9/Cas-L was 1 of 14 cDNAs identified in this screen, and the HEF1/NEDD9/Cas-L mRNA was shown to be abundant in the developing nervous system or in regions populated by neural crest cells, which arise from the lateral edges of the neuroepithelium. The HEF1/NEDD9/Cas-L mRNA was found in the early hindbrain, prior to the development of rhombomeres, and in the developing spinal cord associated with the proliferating neuroepithelium: this localization is highly suggestive of a role of NEDD9 in nervous system development. Moreover, the exposure of rat embryos to excess atRA between E9.25 to E12 lead to altered HEF1/NEDD9/Cas-L expression in the hindbrain within 6 hours after treatment. HEF1/NEDD9/Cas-L expression was also perturbed in vitamin A-deficient embryos. Finally, a RXR/RAR heterodimer specifically bound the HEF1/NEDD9/Cas-L promoter region, suggesting this transcription factor may directly regulate HEF1/NEDD9/Cas-L.

Based on this work, upregulation of HEF1/NEDD9/CAS-L may be an important means whereby atRA promotes cell spreading and neurite outgrowth. Suggestively, HEF1/NEDD9/Cas-L has also been shown by another group to interact with a novel protein, MICAL (molecule interacting with CasL). MICAL is a mediator of Plexin-A activation, which is required for Semaphorin3A signaling, which provides diffusible and repellant axonal guidance cues during nervous system development (122,123). HEF1/NEDD9/Cas-L has also been reported to be transcriptionally upregulated in the dendrites and cytosol of neurons in the cerebral cortex and hippocampus from 1 to 14 days after global ischemia in rats (42); it was subsequently established that HEF1/NEDD9/Cas-L overexpression can promote neurite outgrowth of PC-12 cells. Taken together, these results suggest that HEF1/NEDD9/Cas-L status will prove to have an important role in brain development and/or development of pathological conditions affecting the brain.

HEF1/NEDD9/Cas-L has also appeared as part of a signature of male-overexpressed, sexually dimorphic genes identified in a recent study of gonadal differentiation in mice (124). Nef et. al. used microarray analysis to perform a large-scale transcriptional analysis of XX and XY Sf1-positive gonadal cells during sex determination, confirming results by RT-PCR and/or whole mount in situ hybridization with XX and XY gonads between E10.5-E13.5. Along with HEF1/NEDD9/Cas-L, this study identified a number of other male-overexpressed genes associated with the differentiation and function of male gonads. Whether HEF1/NEDD9/Cas-L plays any role in sexual differentiation remains to be established. However, the fact that HEF1/NEDD9/Cas-L has previously been shown to bind and functionally interact with the Id family of differentiation-regulating transcription factors is intriguing in this and potentially other contexts for control of organismal development (19).

Perspectives for cancer treatment

What might we conclude about the role of HEF1/NEDD9/Cas-L in metastasis, and the features that cause HEF1/NEDD9/Cas-L to be a pro-metastatic factor? HEF1/NEDD9/Cas-L is a scaffolding protein; its role in normal cells is to connect upstream inputs to downstream

effectors. HEF1/NEDD9/Cas-L expression and activation are regulated by cell cycle and by multiple cues provided by receptors for diffusible and attachment-related stimuli, and relocates from focal adhesions to the mitotic apparatus. Because of this complex “hub” function, elevated HEF1/NEDD9/Cas-L expression is poised to influence the cell growth controls in many different ways (Figure 6). Within a normal cell or an early tumor, elevating intracellular HEF1/NEDD9/Cas-L would increase cell migration and invasion, but also trigger post-mitotic defects associated with failed cytokinesis, and undergo processing to fragments that might cause de-adhesion and help promote anoikis. However, the aggressive tumors characterized by HEF1/NEDD9/Cas-L overexpression have extensive prior lesions, including activation of Ras and inhibition of p16Ink4, translocations to produce BCR-ABL, or transformation with HTLV-1 (1,3,61,109). Such prior changes would provide sustained anti-apoptotic signaling, while inactivating cell division checkpoints, allowing tumors to exploit the pro-invasive activities also associated with HEF1/NEDD9/Cas-L.

Does HEF1/NEDD9/Cas-L contribute in the same way to cancer progression in different classes of tumors, and have all potential pro-tumor activities of HEF1/NEDD9/Cas-L been identified? There are fundamental differences between solid and hematopoietic tumors. For example, blood cells infiltrate other tissues as part of their normal function, while epithelial cells are static. Hence, it is reasonable to suppose that while a HEF1/NEDD9/Cas-L-dependent increase the invasive potential of epithelial cells may define an important contribution to the metastasis of solid tumors, this aspect of HEF1/NEDD9/Cas-L function may be less critical in the expansion of BCR-Abl dependent tumor populations. Most of the previous discussion of HEF1/NEDD9/Cas-L function has focused on the “first stages” of the metastatic process, i.e., the escape of tumor cells from their local environments. It is also conceivable that elevated and activated HEF1/NEDD9/Cas-L promotes the later stages of successful metastasis. For melanoma, HEF1/NEDD9/Cas-L overexpression has been associated with formation of metastases in the lung: it is not yet clear whether elevation of HEF1/NEDD9/Cas-L may contribute to metastasis to other tissues. Integrin-associated signaling effectors have been shown to participate in “inside-out” signaling processes, which induce changes in the affinity of the transmembrane integrin protein for specific extracellular ligands. HEF1/NEDD9/Cas-L may, through inside-out signaling, induce the expression or activation of specific “homing receptors” which could include integrins and other transmembrane receptors, such as the CXCR4 chemokine receptor, that allow targeting of metastatic tumors to new microenvironments rich in their cognate ligands. All of these points require further investigation.

Why has HEF1/NEDD9/Cas-L been identified as a pro-metastasis gene, but not its much-studied family member p130Cas? It is perhaps significant that the expression of HEF1/NEDD9/Cas-L is dynamically regulated in normal cells. Levels of HEF1/NEDD9/Cas-L fluctuate throughout the cell-cycle (21,29,83) based on changes in transcription and proteasomal degradation, as discussed above. Such tight regulation of the expression levels of this molecule provides a mechanism to reconcile the apparently disparate functions of HEF1/NEDD9/Cas-L in cell cycle, migration and apoptosis in normal cells: i.e., high levels of HEF1/NEDD9/Cas-L are present in G2/M, but limited in other cell cycle compartments. Because HEF1/NEDD9/Cas-L is dynamically regulated, lesions targeting aspects of its control system can be readily targeted to increase HEF1/NEDD9/Cas-L levels in tumor cells when its expression is favored. In contrast, p130Cas characteristically has stable and ubiquitous expression that may not offer many control points for further upregulation. Moreover, to date, no p130Cas actions at the centrosome regulating mitosis, or promoting apoptosis, have ever been described: hence, there would be no reason to limit upregulation of p130Cas (if possible) to a late stage in tumor progression. Interestingly, one set of studies has associated increased levels of p130Cas with poor prognosis in breast cancer, but in contrast to data with HEF1/NEDD9/Cas-L, the increase

was not dramatic, and was a predisposing factor found in early tumors, rather than a late-stage event (125,126).

To date, most studies of HEF1/NEDD9/Cas-L bearing on metastasis have focused on the cell autonomous role of this protein within cultured cell lines or in tumors. It is also possible that changes in HEF1/NEDD9/Cas-L in non-tumor cells may prove to be important. In an interesting report, HEF1/NEDD9/Cas-L has been shown to be among the downstream effectors of Pyk2-regulated angiogenesis (127). Besides its role in development, efficient angiogenesis is essential for tumor progression (127). This study indicated that Pyk2 tyrosine kinase activity was essential for the pulmonary vascular endothelial cell spreading, migration, morphogenesis, as well as pulmonary vein and artery angiogenesis, and that Pyk2 regulation of p130Cas and HEF1/NEDD9/Cas-L was specifically important for these processes. The relevance of HEF1/NEDD9/Cas-L-dependent angiogenesis to tumor progression and metastasis in vivo has not yet been addressed.

How might HEF1/NEDD9/Cas-L be exploited to improve anticancer therapy? On a fundamental level, better knowledge of HEF1/NEDD9/Cas-L action in normal cells and tumors should improve our understanding of how tumors pass from localized and readily controlled, to metastatic and refractory to therapy. Should the role of HEF1/NEDD9/Cas-L at metastasis be explicitly related to its multiple functions in migration, cell cycle and apoptosis, this may nominate other genes with similar properties as candidates for scrutiny. A growing number of proteins are now being appreciated to connect different aspects of HEF1/NEDD9/Cas-L (128); some of them, such as adenomatous polyposis coli (APC), are already well established as tumor-relevant targets. Because it apparently lacks catalytic activity, HEF1/NEDD9/Cas-L is not immediately promising as a target for directed drug development, unless this be through agents intended to disrupt its protein-protein interactions, or through an siRNA-based approach to globally deplete HEF1/NEDD9/Cas-L levels. If HEF1/NEDD9/Cas-L-directed drugs or siRNAs should be developed, it is encouraging that the genetic HEF1/NEDD9/Cas-L knockout animal has relatively limited defects, as this implies loss of HEF1/NEDD9/Cas-L can be well tolerated. As an alternative strategy, given the dependence of HEF1/NEDD9/Cas-L overexpression on pre-existing lesions such as upregulation of Ras, the use of compounds designed to inhibit Ras-pathway signaling (129) or BCR-ABL (130) may be particularly effective in metastatic tumors characterized by elevated HEF1/NEDD9/Cas-L expression. Use of such reagents might eliminate the pro-survival signaling necessary to tolerate elevated HEF1/NEDD9/Cas-L levels. The availability of HEF1/NEDD9/Cas-L knockout mice should contribute significantly to such investigations. Currently, there are no reports in the literature of a transgenic HEF1/NEDD9/Cas-L mouse model; once such a model is generated, it should be a vital reagent for studies both of development and cancer.

Summary

Together, the studies of HEF1 molecular regulation and function have raised a number of interesting questions regarding the potential role for HEF1 in metastasis. First, in what ways does elevated HEF1/NEDD9/Cas-L expression promote the metastatic process? Second, why are increased levels of HEF1/NEDD9/Cas-L observed at late stages of tumor progression, rather than during tumor initiation? Third, is there any way to exploit the defined biology of HEF1/NEDD9/Cas-L to limit tumor cell metastasis? Fourth, might altered HEF1/NEDD9/Cas-L action be relevant to other diseases or developmental disorders? ***The model we propose*** based on the data presented above is that HEF1/NEDD9/Cas-L is a central coordinator of cell migration, apoptosis, cell cycle, and other signaling processes. While some of the consequences of elevated HEF1/NEDD9/Cas-L expression are beneficial for tumor growth, others are inhibitory, and hence high HEF1/NEDD9/Cas-L levels can only be tolerated subsequent to tumor acquisition of enabling genetic or epigenetic modifications.

Over the past ~ 13 years of investigations into the Cas family of proteins, the chief molecule of interest for this group has been the first described family member, p130Cas. For many protein families, it is often assumed that paralogous proteins have comparable function, and that separate study of subsequent family members after functional definition of a first will generally reveal limited differences. For HEF1/NEDD9/Cas-L, this is clearly not the case. Based on the exciting research summarized in the present review, we predict that HEF1/NEDD9/Cas-L will emerge from the shadow of its sibling, and subsequent research will firmly establish the key biological roles for this intriguing molecule, not the least of which is its critical role as a pro-metastatic factor.

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Figure 1. Domain Structure of HEF1/NEDD9/CAS-L

Amino acids encompassing each domain are indicated. See text for details.

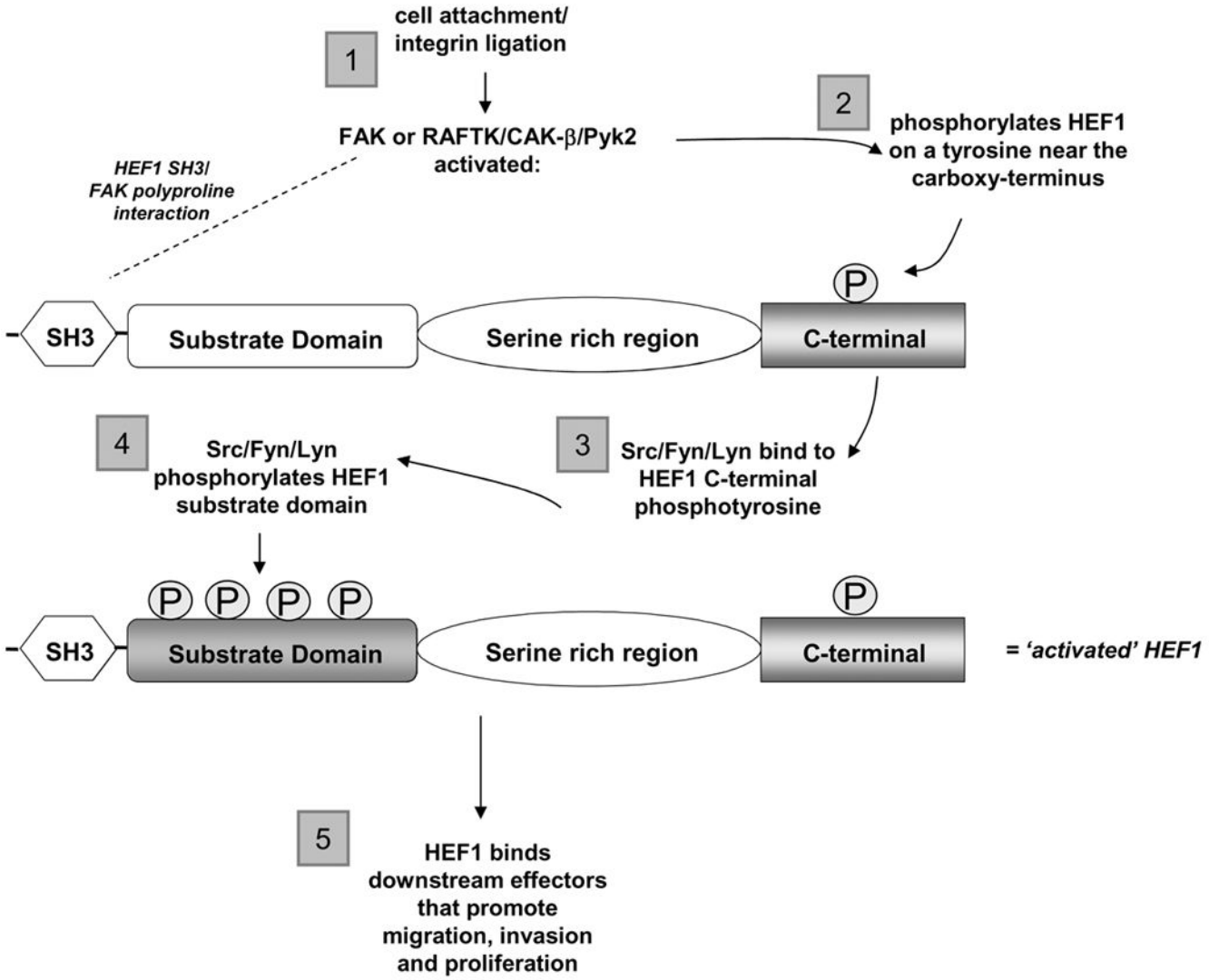

Figure 2. Sequence of protein interactions and post-translational modifications "activating" HEF1/ NEDD9/CAS-L following integrin ligation

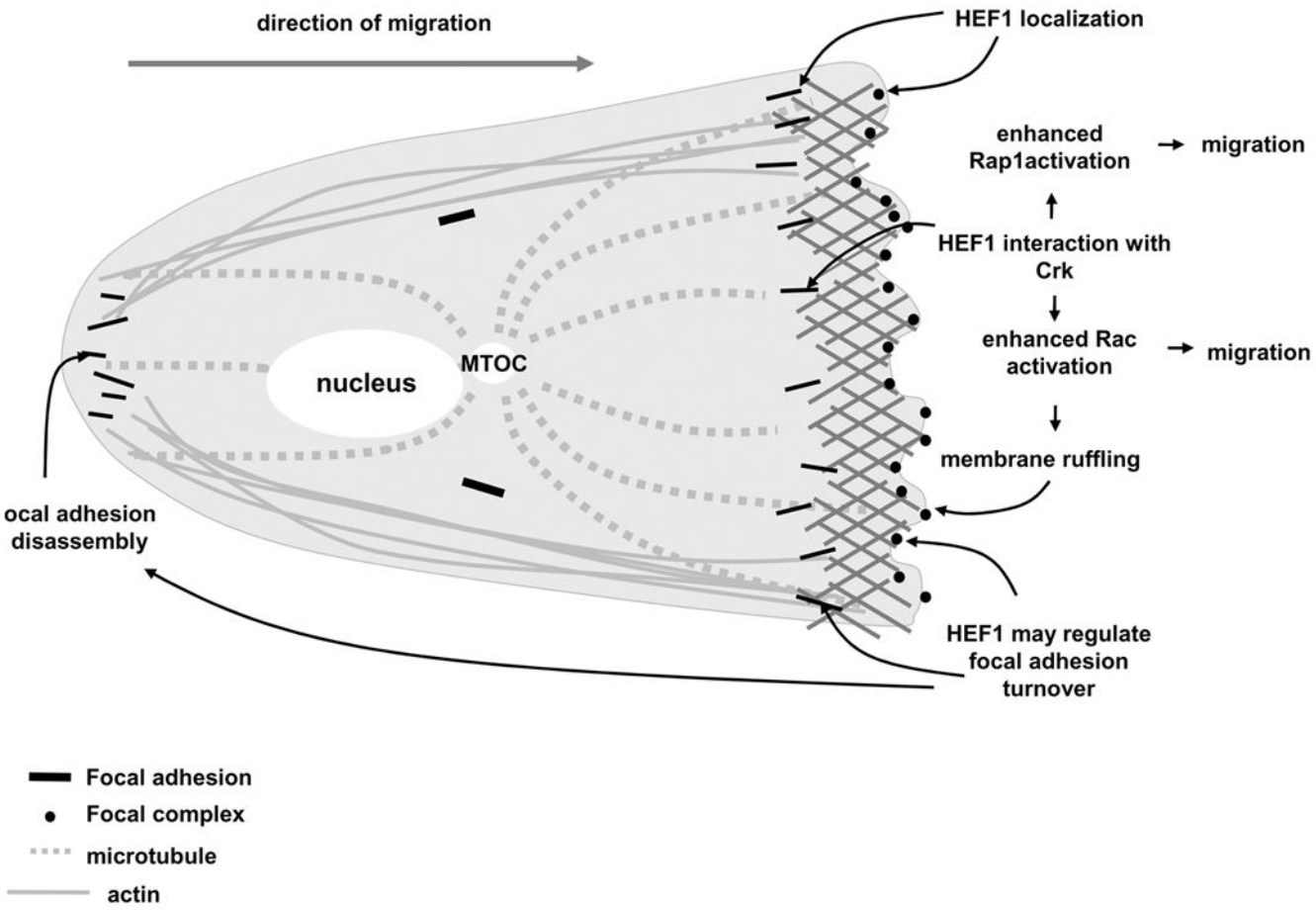

Figure 3. HEF1/NEDD9/CAS-L localization and actions during cell migration

HEF1/NEDD9/CAS-L causes Rac activation in lamellipodia, influencing the dynamics of actin polymerization. HEF1/NEDD9/CAS-L may also contribute to focal adhesion turnover through other protein interactions described in the text.

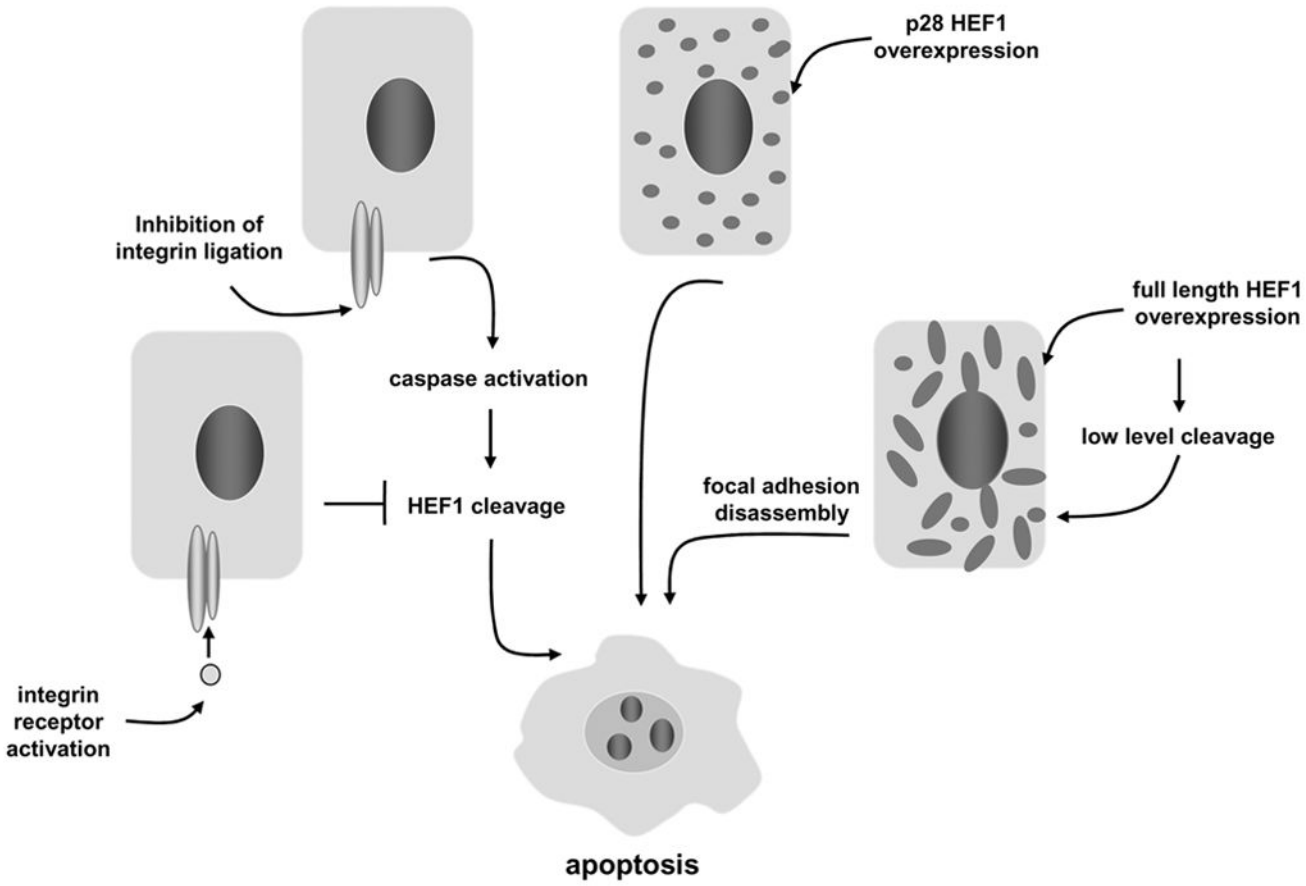

Figure 4. HEF1/NEDD9/CAS-L in apoptosis/anoikis

Cleavage of HEF1/NEDD9/CAS-L by caspases is inhibited by integrin ligation, and promoted by enforced cell detachment. The carboxy-terminal cleaved fragment of HEF1/NEDD9/CAS-L (p28) actively promotes focal adhesion disassembly and cell death.

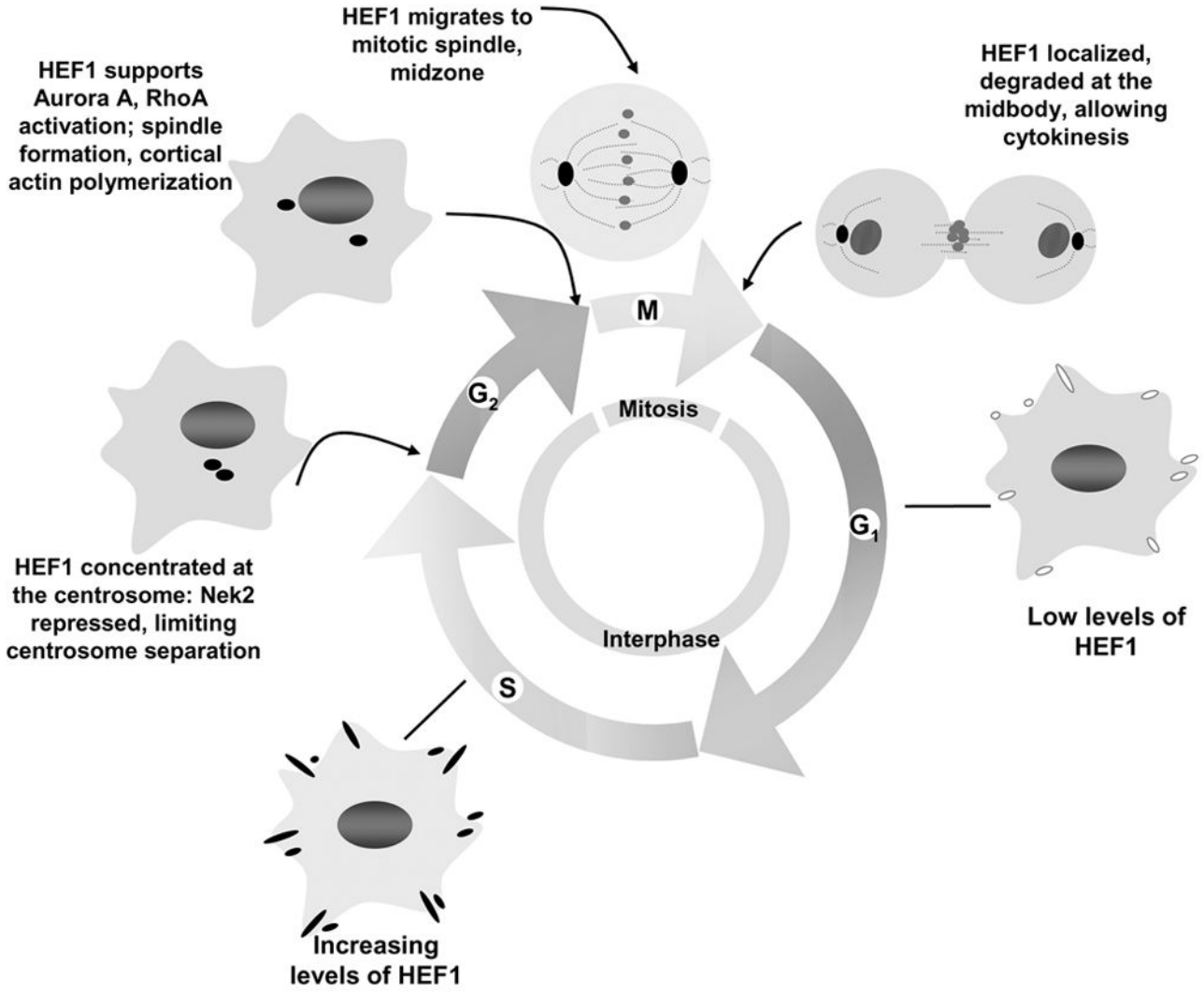

Figure 5. HEF1/NEDD9/CAS-L regulation and action through cell cycle

HEF1/NEDD9/CAS-L levels increase through S phase, peaking in G2/M. HEF1/NEDD9/CAS-L relocalizes to centrosomes, and activates Aurora A and RhoA in mitosis.

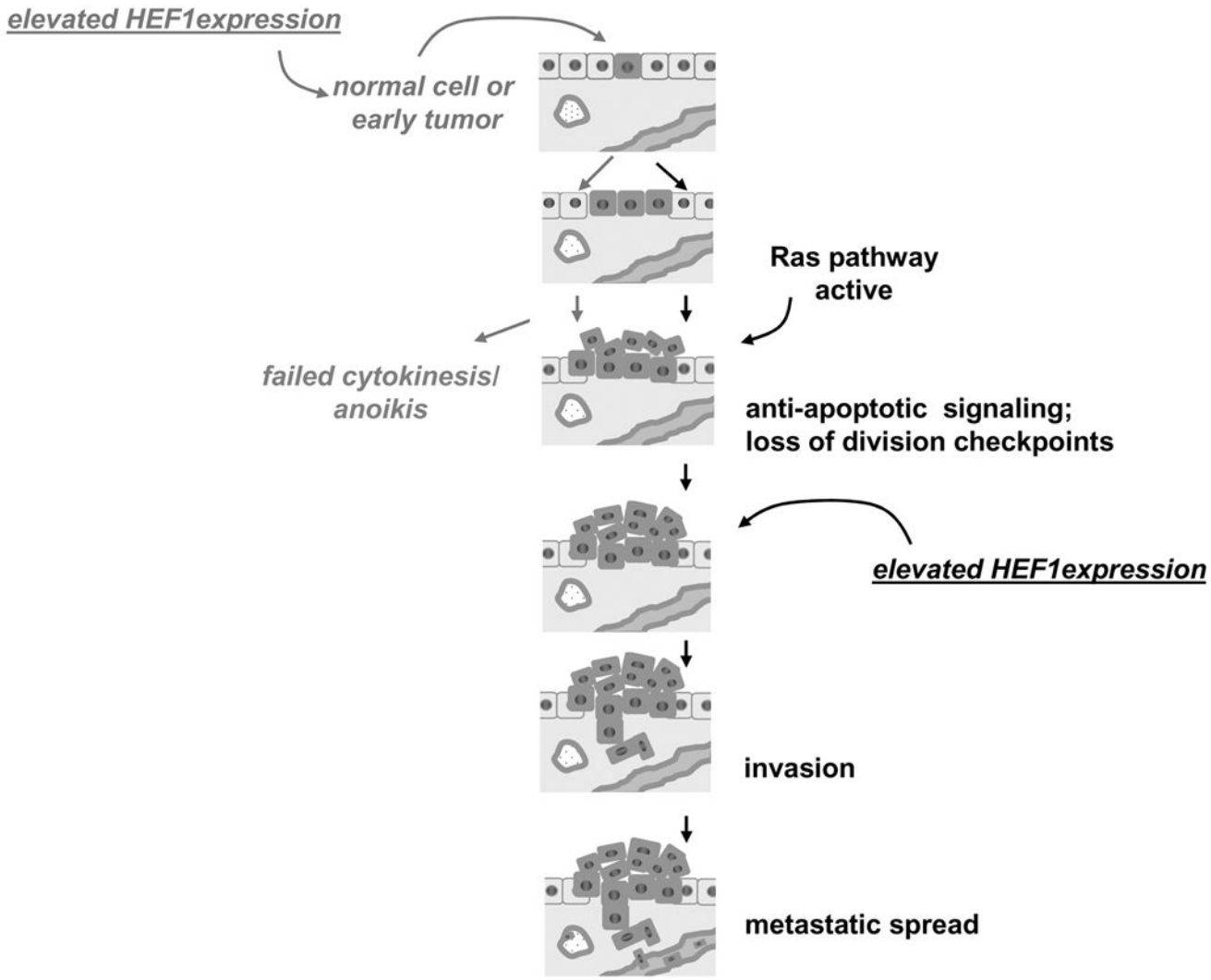

Figure 6. Model for HEF1/NEDD9/CAS-L in metastasis
See text for details.