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Molecular basis for HEF1/NEDD9/Cas-L action as a multifunctional co-ordinator 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 · HEF1 · 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.

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

The contribution of Mahendra K. Singh and Lauren Cowell was equivalent.

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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 Downregulated [4]. 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 co-ordinately regulate cell polarization and cell cycle in human cells [5]. 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 [5].

Also in 1996, Minegishi et al. identified Cas-L (for Crk-associated substrate-related protein, Lymphocyte type) [6], 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 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 Bouton et al. and DeFilippi 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 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 SH_3 domain, which confers interactions with partner proteins bearing a poly-proline motif [15]. Amino acids ~90–350 are characterized by a large number of SH_2 binding sites: tyrosine-containing motifs that when phosphorylated allow binding to partner proteins bearing SH_2 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 SH_3-domains in partner molecules, but these poly-proline stretches are absent from HEF1/NEDD9/Cas-L.TPB -.2

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 kDa and a 115 kDa protein [29], significantly larger 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

Fig. 1 Domain Structure of HEF1/NEDD9/Cas-L. Amino acids boundaries of each domain are indicated. See text for details

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 Fig. 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 hyperphosphorylated 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 115 kDa 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 disruptions of microtubule and intermediate filament networks do not influence the p105/p115 ratio [36]. Bargon and coworkers also demonstrated

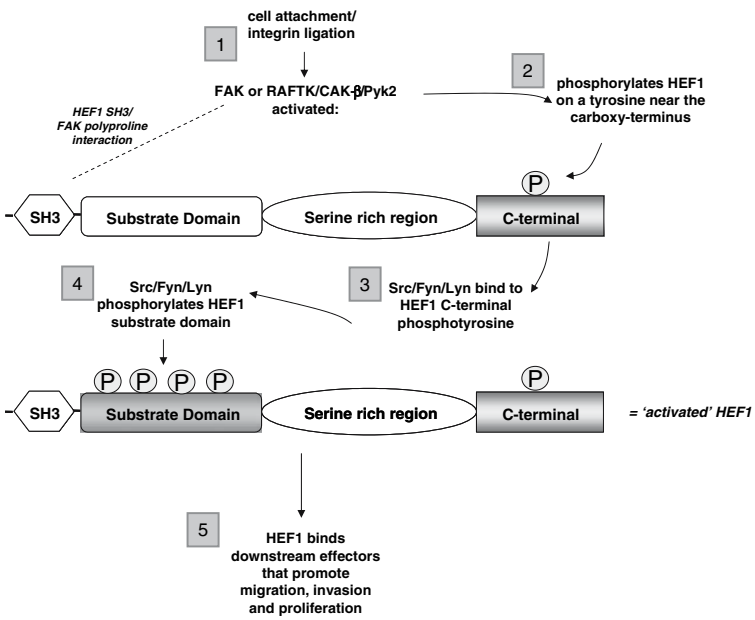

Fig. 2 Sequence of protein interactions and post-translational modifications ''activating'' HEF1/NEDD9/Cas-L following integrin ligation

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 p115 kDa isoform of HEF1/NEDD9/Cas-L whereas the p105 isoform is largely unaffected: in cells that were allowed to establish focal adhesions prior to serum starvation, the p115 kDa 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 ischemia 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 (atRA) 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 upregulated in response to progesterone receptor A overexpression [45], but has been reported to be downregulated in ER-α-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 ischemia 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 co-ordination 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 co-ordinating 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 (Fig. 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 pro-migratory 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

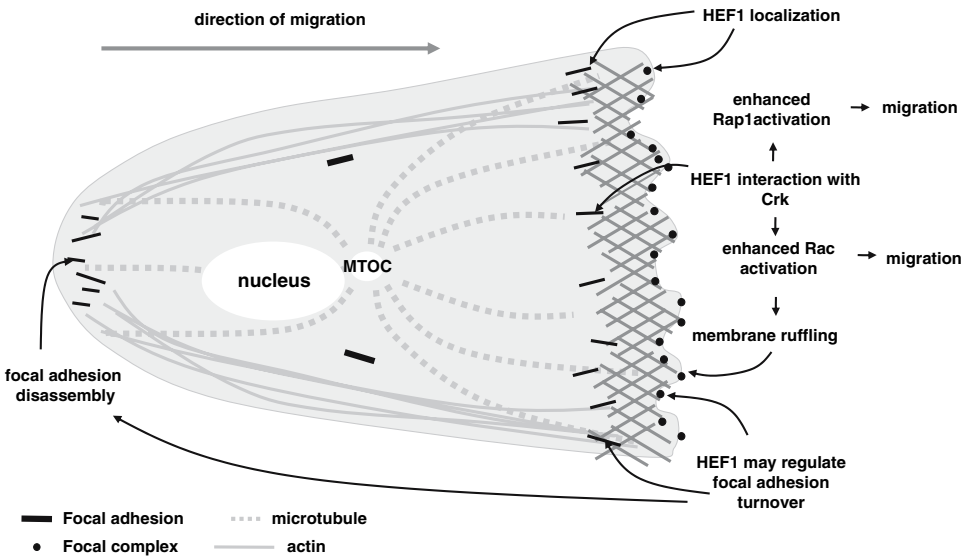

Fig. 3 HEF1/NEDD9/Cas-L localization and actions during cell migration. HEF1/NEDD9/Cas-L causes Rac activation in lamellipodia, influencing the dynamics of actin polymerization. HEF1/NEDD9/Cas-L may also contribute to focal adhesion turnover through other protein interactions described in the text

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 depend 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 knockdown 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 cannot compensate for HEF1/NEDD9/Cas-L migratory function at least in this particular tissue [55]. Notably, Rho kinase inhibition [37], and FAK knockdown 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 by induced expression of HEF1/NEDD9/Cas-L, 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 signaling pathways, extracellular 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 co-ordinated via the activation of caspases, enzymes that cleave proteins. Cells undergoing apoptosis exhibit characteristic morphological features of cell rounding and membrane blebbing, and disassemble focal adhesions. Caspase cleavage of proteins during apoptosis can 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 (Fig. 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 2 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.

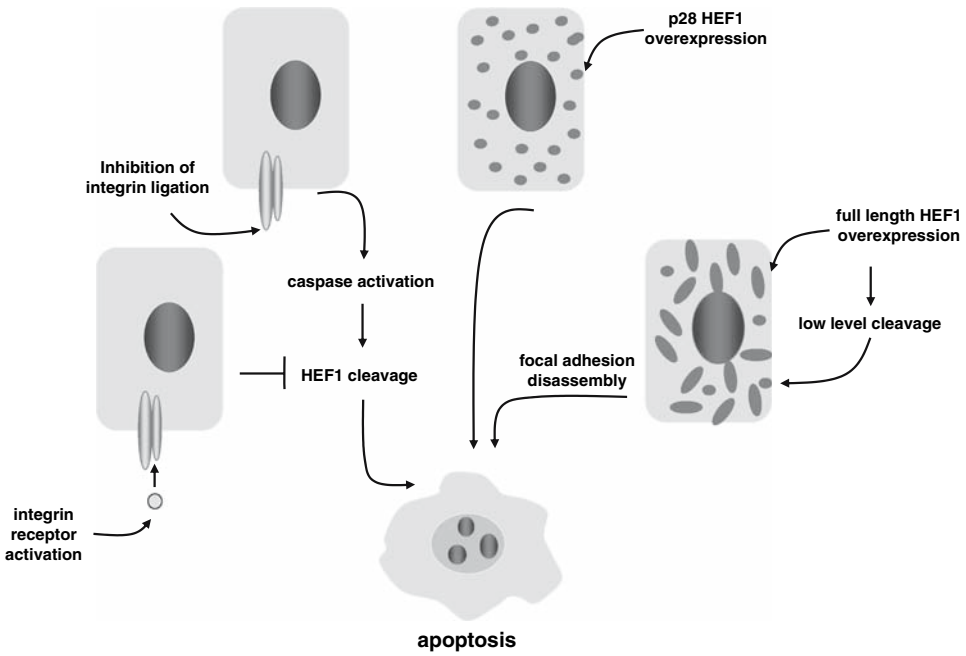

Fig. 4 HEF1/NEDD9/Cas-L in apoptosis/anoikis. Cleavage of HEF1/NEDD9/Cas-L by caspases is inhibited by integrin ligation, and promoted by enforced cell detachment. The carboxy-terminal cleaved fragment of HEF1/NEDD9/Cas-L (p28) actively promotes focal adhesion disassembly and cell death

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 (Fig. 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].

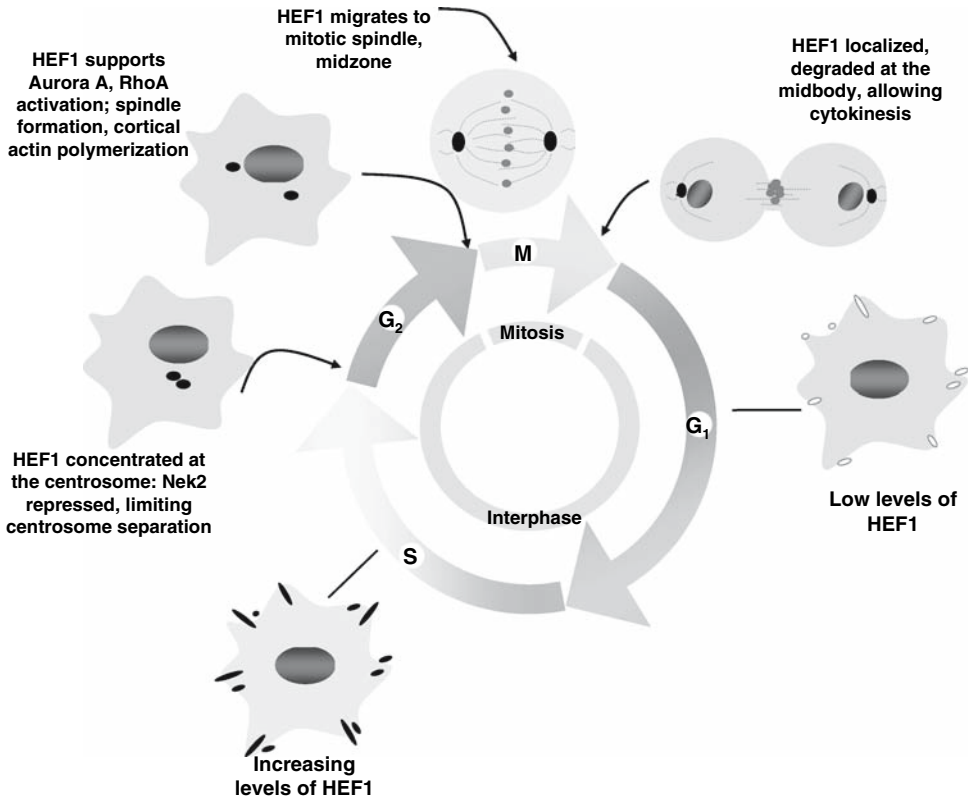

Fig. 5 HEF1/NEDD9/Cas-L regulation and action through cell cycle. HEF1/NEDD9/Cas-L levels increase through S phase, peaking in G2/M. HEF1/NEDD9/Cas-L relocalizes to centrosomes, and activates Aurora A and RhoA in mitosis

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 co-ordinator 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 hematopoietically expressed paralog of the ubiquitously expressed CHAT (Cas/HEF-associated signal transducer, 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 co-ordinate 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 (ATL) 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 HTLV1 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-κB [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-κB 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 HEF1/NEDD9/Cas-L 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 and 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. 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 (Fig. 6). 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 HTLV1 [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

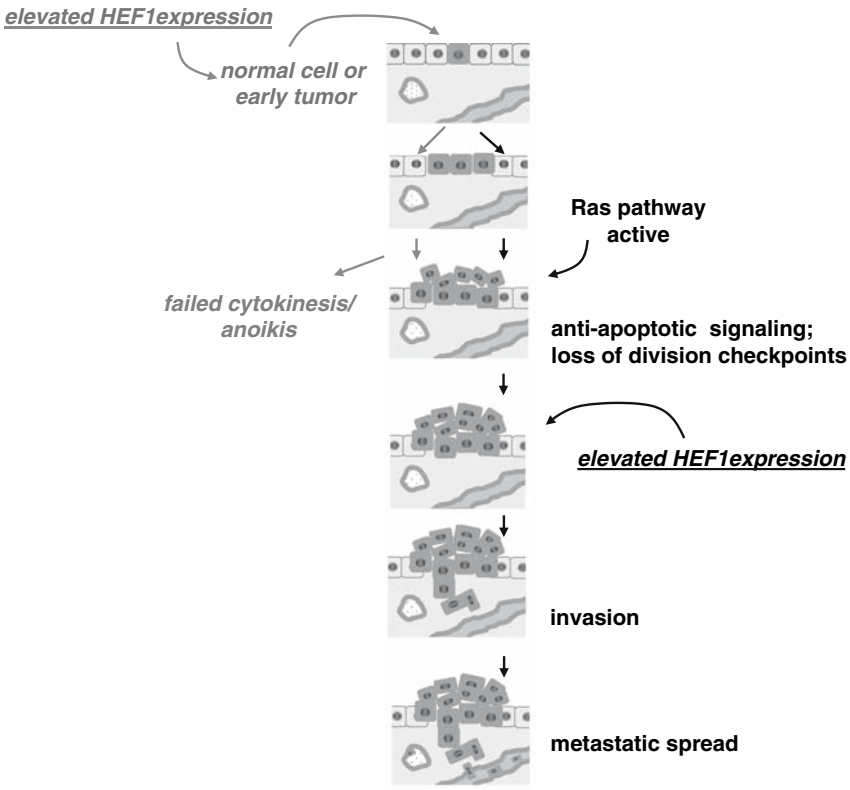

Fig. 6 Model for HEF1/NEDD9/Cas-L in metastasis. See text for details

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. Since, it apparently lacks catalytic activity, HEF1/NEDD9/Cas-L is not immediately promising as a target for directed drug development, unless this is 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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