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The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome

Elena N. Pugacheva and Erica A. Golemis*

Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111

Abstract

Although HEF1 has a well-defined role in integrin-dependent attachment signaling at focal adhesions, it relocates to the spindle asters at mitosis. We report here that overexpression of HEF1 causes increase in centrosome numbers and multipolar spindles, resembling defects induced by manipulation of the mitotic regulatory kinase Aurora A (AurA). We show that HEF1 associates with and controls activation of AurA. We also show HEF1 depletion causes centrosomal splitting, monoastal spindles, and hyperactivation of Nek2, implying additional action earlier in cell cycle. These results provide new insights into the role of an adhesion protein in coordination of cell attachment and division.

Keywords

HEF1; centrosome; spindle; mitosis; Aurora-A; Nek2

HEF1 is a member of a group of scaffolding proteins that includes p130Cas and Efs/Sin^{1,2}. This group of Cas proteins localizes to focal adhesions in interphase cells, and acts as intermediates in a variety of integrin-dependent signaling processes, including establishment of cell attachments, migration, and cell survival signaling. In 1998, we proposed that HEF1 might have a previously unsuspected function in mitosis³, based on the observation that the HEF1 protein relocated from focal adhesions to the mitotic spindle asters in M-phase. Since that time, reports have appeared suggesting of the association of other focal adhesion complex proteins, such as zyxin⁴, paxillin⁵, FAK, and Pyk2⁶ with the mitotic spindle or other relevant structures such as the microtubule organizing center (MTOC) or centrosome. Recent work has emphasized the dual activity of centrosomes in contributing to control of cell polarization in interphase cell migration^{7,8}, but also in coordinating assembly of the mitotic spindle in M-phase⁹. Centrosomally-associated signaling activities such as the Aurora-A (AurA) kinase also govern the timing of mitotic entry¹⁰, for instance by regulating the activation of cyclin B1¹¹. In this study, we demonstrate an requirement for HEF1 in activation of AurA and Nek2¹², a second kinase important for centrosome cohesion, and we provide additional data indicating HEF1 provides a crucial bridge coordinating cell attachment and cell division processes in mammalian cells.

Results

Cell cycle-regulated HEF1 localization

HEF1 localizes to the centrosome of MCF7 cells in a cell cycle regulated manner (Figure 1A), with centrosomal signal lowest in G1, and strongest in G2/M cells. This corresponds to

* corresponding author: Erica Golemis, W406, Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111, Phone: 215-728-2860, Fax: 215-728-3616, Email: EA_Golemis@fccc.edu.

relatively low levels of HEF1 detectable in G1, and the fact that the bulk of HEF1 in interphase cells localizes to focal adhesions (Figures 1B, 1C; Supplemental Figure 2A; 3). As HEF1 levels increase during G2, a slower migrating, hyperphosphorylated species becomes more apparent, as we have previously reported³. At mitotic entry, a significant fraction of HEF1 moves to the spindle, and HEF1 is no longer detectable at the centrosome at cytokinesis. The endogenous HEF1 localization pattern described here was lost following HEF1 depletion with siRNA, supporting signal specificity (Supplemental Figure 1A, B). Further, a mouse monoclonal antibody newly generated to HEF1, mAb-14A11, and transiently over-expressed GFP-fused HEF1, generated the same pattern (Supplemental Figures 1C, 1D, and 2A). Finally, HEF1 signal in the vicinity of the centrosome was coincident with the patterns seen for multiple centrosome-associated proteins, including γ -tubulin, c-Nap1, pericentrin, ninein, and Nek2 (Supplemental Figure 2B). Using GFP- and FLAG epitope-fused HEF1 derivatives we mapped minimal sequence determinants necessary for localization of HEF1 to the centrosome as HEF1 amino acids 1-405 (Figure 1 D, E), containing the SH3 domain and SH2-binding site-rich domains², with sequences between 363–405 an essential localization determinant. In addition to this fragment, carboxy-terminal derivatives of HEF1 (aa 351-653, and 654-834) also showed weaker association with the centrosome, suggesting more than one interaction contributes to the localization, analogous to the discrete focal adhesion targeting elements within HEF1¹³.

Up- or down-regulation of HEF1 expression causes centrosomal and spindle abnormalities

To determine the functional significance of HEF1 association with the centrosome and the mitotic spindle, we established three independent systems for manipulation of HEF1 in MCF-7 cells. First, we overexpressed the full length HEF1 protein under control of a tetracycline-repressible promoter (Figure 2A). Second, based on prior data indicating HEF1 cleavage and proteolysis at mitosis and apoptosis (discussed further below), we stabilized full length endogenous HEF1 using peptide aptamers targeted to a previously mapped HEF1 cleavage site^{13,14} (Figure 2B). Third, we used an siRNA approach to deplete endogenous HEF1, using two independent siRNAs (siHEF1, and siHEF1a: Figure 2C and Supplemental Figure 3A). For each manipulation, a matching negative control set was used, corresponding to tetracycline-regulated GFP (for overexpression), use of non-specific peptides, and use of a scrambled siRNA, a GFP-targeted siRNA, and in some cases a p130Cas-targeted siRNA for depletion (Figure 2C and Supplemental Figure 3A).

Both overexpression of exogenous HEF1 and peptide stabilization of endogenous HEF1 induced a high frequency of cells with spindle defects by 48 hours following cell treatment (Figure 3A, B). Most notable was the increase in the number of cells with multipolar spindles, which represent 12% of the mitotic cell population with overexpression, and >16% with specific HEF1-targeted peptides, as compared to 2–3% for all negative controls. For every cell with multipolar spindles, each spindle originated from a γ -tubulin, pericentrin or GFP-centrin positive structure (Figure 3A, Supplemental Figure 4A and not shown). In other cells, while no multipolar spindles were observed, nevertheless the spindle was defective. A common phenotype was the presence of “bent” spindles (Figure 3A(– tet)), which would arise if the spindle poles had not fully moved to opposite sides of the cell. Overexpression of HEF1, or introduction of HEF1-stabilizing peptides, was also found to consistently induce supernumerary centrosomes, with >10% of all cells containing in excess of 3 centrosomes. (Figures 3A, C, and Supplemental Figure 4). Abnormally increased numbers of centrosomes can arise from either deregulation of the centrosomal duplication cycle during S phase, or as a result of failed cytokinesis¹⁵. We found that the increased numbers of centrosomes accumulated gradually over 24–48 hours following HEF1 induction (Figure 3C), and were due to secondary defects in cytokinesis, because centrosomes did not accumulate in HEF1-overexpressing cells held in hydroxyurea (Figure 3D).

In contrast to the results with overexpressed HEF1, depletion of HEF1 induced a high percentage of cells with monoastral spindles, containing two γ -tubulin-positive structures (Figure 4A, center row), or with poorly formed spindles (Figure 4A, bottom row). The centrosomes of cells with defective spindles showed weaker reactivity with antibody to γ -tubulin than did cells with undepleted HEF1 (Figure 4A). Further, the spindles consistently were less reactive with α -tubulin antibody, particularly in cells with less well-formed spindles (Supplementary Figure 1B). Parallel staining was done with antibody to HEF1 indicated that HEF1 itself was effectively depleted in individual cells with noticeable phenotypes (Supplemental Figure 3B, bottom row): the most pronounced phenotypes were observed in cells with the greatest degree of HEF1 depletion. HEF1-depleted non-mitotic cells also manifested centrosomal abnormalities. Two distinct, widely separated, GFP-centrin-positive structures (Figure 4B, and Supplemental Figure 3B) were observed in >70% of HEF1-depleted cells but in 25–30% of cells treated with a scrambled siRNA (Figure 4C). Normally, two widely separated centrosomes are not observed until the G2/M transition^{15,16}. This increase in the frequency of split centrosomes was observed with both HEF1-directed siRNAs, but with neither of the two non-specific siRNAs; a weaker effect was seen with a p130Cas-directed siRNA (Figure 4C). FACS analysis of HEF1-depleted cells versus scrambled siRNA-treated controls confirmed that their cell cycle profile does not show G2 enrichment (Figure 4D). Hence, the primary defect with HEF1 depletion is likely one of centrosome cohesion, resulting in premature splitting, rather than a secondary consequence of altered cell cycle compartmentalization.

HEF1 interacts with AurA and is required for the activation of AurA kinase

The phenotypes described above for HEF1 depletion and overexpression are similar to those reported for inhibition or overexpression of other proteins known to regulate centrosomal maturation and cell cycle progression, including notably the AurA kinase^{17,18}. Indeed, while in HEF1-depleted MCF-7 cells in the G2 phase of cell cycle, total levels of AurA at the centrosome were similar to those found in MCF-7 cells treated with a matched scrambled siRNA (Figure 5A, B), in contrast, levels of phospho-AurA (T288: indicative of kinase activation¹¹), were greatly reduced or absent under conditions of HEF1 depletion (Figure 5A, B). This implied that HEF1 plays an important role in the activation of AurA. This role could be direct, with HEF1 physically a component of an AurA activation complex, or it might be indirect, with HEF1 causing defects at an early stage of the centrosomal cycle that interfere with AurA activation. AurA and HEF1 co-precipitated from whole cell lysates, with greatest levels of association in cells in G2/M (Figure 5C), suggesting HEF1 control of AurA activation might be direct, through physical interaction with AurA itself, or an AurA-containing complex.

Using a series of GST-fused truncations of HEF1, we determined that GST-HEF1₁₋₃₆₃ was the minimal sequence required to efficiently pull down AurA *in vitro* (Figure 6A). Our earlier results (Figure 5A, B) predicted that HEF1 interaction with AurA might help to activate the kinase. Full length HEF1 is not stable as a recombinant purified protein, prohibiting a direct *in vitro* test of this idea with the native protein (results not shown). However, as an alternative approach, we titrated the GST-HEF1₁₋₃₆₃ minimal AurA-interacting domain versus GST into an *in vitro* kinase reaction containing recombinant AurA purified from bacteria (Figure 6B). Increasing levels of GST-HEF1₁₋₃₆₃, but not of GST, clearly induced the auto-phosphorylation of AurA, and the ability of AurA to phosphorylate a histone H3 substrate, indicating that the association with HEF1 is sufficient to promote AurA catalytic activity¹¹. Indeed, a higher level of activated AurA was observed in cells overexpressing HEF1, opposite to the lower levels of AurA activation seen with HEF1 depletion (Figure 6C). Further, we found that both GST-HEF1₁₋₃₆₃ and HEF1₁₋₄₀₅ were phosphorylated by recombinant activated AurA *in vitro* (Figure 6D).

Although the AurA consensus site remains poorly defined, an RHQS₂₉₆LSP motif closely resembles a site phosphorylated by the Aurora yeast ortholog Ipl1p¹⁹. We used mass spectrometry analysis of *in vitro* phosphorylated GST-HEF1₁₋₃₆₃ to confirm *in vitro* phosphorylation of this site (not shown), and mutated S₂₉₆ into alanine (unphosphorylatable) or glutamic acid (mimicking constitutive phosphorylated HEF1) alone or together with an adjacent serine, S₂₉₈. All S₂₉₆ mutants were no longer phosphorylated by AurA (Figure 6E). However, while alanine mutants of GST-HEF1₁₋₃₆₃ maintained the ability to interact with AurA and activate the kinase, glutamic acid mutants of HEF1 lost both abilities (Figure 6F). In parallel, we compared the relative interaction of HEF1 with AurA in the presence or absence of ATP *in vitro* (Supplementary Figure 6A). We found that AurA co-immunoprecipitated much more efficiently with unphosphorylated HEF1, supporting results with the mutants.

In vivo, we then compared the ability of GFP-fused HEF1, HEF1_{S296E}, HEF1_{S296E,S298E}, and HEF1_{S296AS298A} to immunoprecipitate AurA (Figure 6G). While HEF1_{S296AS298A} was similar to HEF1 in interacting with AurA, both phosphomimic variants were severely impaired for AurA interaction, similar to the *in vitro* results. Together, these data suggest a model in which an initial interaction of HEF1 with AurA prior to mitotic entry activates AurA, which then phosphorylates HEF1, promoting dissociation of the two proteins. Amino acids 1–405 are a minimum determinant of strong HEF1 association with the centrosome *in vivo* (Figures 1D, E), with a critical localization determinant located in the serine-rich amino acids from 363–405. We transfected GFP-fused truncation derivatives of HEF1 into the MCF7 cells, immunoprecipitated with antibody to GFP, and confirmed that GFP-HEF1₁₋₄₀₅ coimmunoprecipitated with AurA from whole cell lysates, while GFP-HEF1₁₋₃₆₃ did not (Supplemental Figure 6B). We next compared the activation of AurA immunoprecipitated from cells expressing HEF1, HEF1₁₋₃₆₃, and HEF1₁₋₄₀₅ (Supplemental Figure 6C). GFP-HEF1₁₋₄₀₅ was like GFP-HEF1 in promoting increased activity of AurA against a histone H3 substrate, while GFP-HEF1₁₋₃₆₃ was not. Together, these results imply that a primary role of aa 363–405 in promoting the HEF1-AurA interaction *in vivo* is through localizing HEF1 to the centrosome, where endogenous AurA concentrates (Figure 5A). We were unable to test if GFP-HEF1₁₋₄₀₅ was similar to GFP-HEF1 in inducing multipolar spindles, because in scrutiny of hundreds of cells, no mitotic cells overexpressing GFP-HEF1₁₋₄₀₅ were ever observed, suggesting that this truncation may be disrupting HEF1-dependent processes prior to mitotic entry.

HEF1 negatively regulates NEK2 and contributes to accumulation of pericentriolar material (PCM)

In normal cells, after telophase, centrosomes mature through cell cycle, accumulating PCM that includes signaling proteins that govern centrosomal duplication and other functions such as microtubule nucleation¹⁵. Cohesion of the centrosomes is maintained by c-Nap-1: levels of c-Nap-1 are reduced 10-fold at the G2/M boundary, with phosphorylation by the Nek2 kinase, and potentially AurA, Cdk1, and Plk1 promoting its removal from the PCM and centrosomal disjunction, allowing formation of a bipolar mitotic spindle^{12,20,21}. Moreover, overexpression of Nek2 induces precocious centrosomal disjunction in interphase¹², while AurA depletion does not (Supplemental Figure 5 A, B, C). We examined the status of Nek2 and c-Nap-1 (Figure 7A), and the centrosomal maturation marker ninein (Supplemental Figure 7) at the centrosome in MCF7/GFP-centrin cells depleted of HEF1. HEF1 depletion reduced the signal intensity of all of these proteins at the centrosome, suggesting a contribution of HEF1 to the stable assembly of proteins with the PCM. We next asked if HEF1 regulated Nek2 activation. Nek2 immunoprecipitated from HEF1-depleted cells was much more active in phosphorylating an MBP substrate than Nek2 from control-depleted cells (Figure 7B). Moreover, overexpression of GFP-HEF1 decreased, while overexpression of GFP-HEF1₁₋₄₀₅ increased, activation of Nek2 relative to background levels in cells expressing GFP.

As in the other assays, GFP-HEF1₁₋₃₆₃ had no activity. Finally, we found that endogenous HEF1 and Nek2 co-immunoprecipitated efficiently and specifically from MCF7 cells (Figure 7C). Together, these data implied that HEF1 contributes to Nek2 inhibition during the normal cell cycle.

Separation of HEF1 actions at the centrosome, and in attachment

The main functions previously ascribed to HEF1 and the Cas family of proteins have been in regulation of cell attachment and motility^{1,2}. We had previously shown that over-expression of HEF1 induced cell spreading^{13,22}, whereas overexpression of the HEF1 carboxy-terminus had a dominant negative function on cell attachment, causing cell rounding¹³. To evaluate whether HEF1 control of cell attachment, and regulation of centrosomal splitting, were linked or separable, GFP-fused HEF1 and HEF1 truncations were transfected into MCF7 and HeLa cells, and the ability of different HEF1 domains to act as dominant negatives by inducing centrosomal splitting versus inhibiting cell attachment was scored (Figures 8A, B, C). Based on this analysis, full length HEF1 weakly induced centrosomal splitting. However, the HEF1₁₋₄₀₅ domain proved to have a potent phenotype, and the HEF1₃₅₁₋₆₅₃ fragment a weaker phenotype, in inducing centrosomal splitting (Fig 8 A), while neither affected the degree of cell spreading (Fig 8 B). Conversely, HEF1₆₅₄₋₈₃₄ induced significant cell rounding (Fig 8B), as reported before, but did not affect centrosomal splitting (Fig 8A). These results indicated that separable HEF1 domains were required for centrosomal and attachment activities.

In further test, we determined the consequences of varying the degree of cellular attachment induced by extrinsic stimuli on HEF1-associated centrosomal phenotypes. Cells with induced overexpressed full length HEF1 (Figure 8 D), depleted HEF1 (Figure 8E), or overexpressed dominant negative HEF1₁₋₄₀₅ (Figure 8 F) were plated on either normal tissue substrates, or on poly-L-lysine, fibronectin, or laminin to increase spreading. Cells plated on the latter three substrates were significantly more spread, and were marked by more pronounced paxillin staining at focal adhesion structures (results not shown). However, in scoring the number of supernumerary centrosomes induced by overexpressed HEF1 (Figure 8 D), or the number of split centrosomes induced by removal or dominant negative blockade of HEF1 function (Figures 8 E, F), the greater attachment status did not affect the observed phenotypes. Together with the earlier results, these findings demonstrate that HEF1-induced cell spreading and enhancement of focal adhesions, do not cause the centrosome abnormalities we have described here; rather, a distinct function of HEF1 is involved.

Discussion

The results of this study for the first time establish HEF1 as a regulator of AurA and Nek2 activation, and of centrosome cohesion and amplification. Proteins initially defined as components of the cell attachment machinery, including APC²³ and Ajuba¹¹, have recently been found to also function in cell cycle controls, and elegant genetic studies in lower eukaryotic models for development such as *C. elegans* (reviewed in^{24,25}) have begun to elucidate a model in which dynamic interconnections between the centrosome and structures at the cell cortex controls the plane of mitotic spindle orientation, and cleavage furrow formation. It is likely that this mechanism will prove to be important for higher eukaryotes as well, given the need of many cells to limit cell division to specific planes (for example, to maintain barrier function). An economical view of cellular function would suggest that the re-use of proteins which govern cell attachment and cytoskeletal dynamics in interphase cells might not only be efficient, but might also provide a means to synchronize changes in cell contacts during the mitotic process. It is possible that the pools of HEF1 used for centrosome, mitotic spindle, and focal adhesions are completely distinct. However, it may be that migration of proteins such as HEF1 between these structures provides polarity and attachment cues that

influence the entry to and exit from mitosis. Given the particular abundance of HEF1 in polarized epithelial and lymphoid cell populations, our work would define it as an excellent candidate for such a role.

Our data suggest a model in which in normal cells, HEF1 initially interacts with AurA in G2 prior to AurA activation, with the centrosome one important point of interaction. In this model, as mitosis approaches, focal adhesion disassembly releases more HEF1, and the increasing interaction of HEF1 with AurA promotes AurA activation. In turn, phosphorylation of HEF1 by the activated AurA reduces the affinity of interaction between the two proteins, perhaps contributing to the relocation of HEF1 away from the centrosome, or perhaps contributing to preferred interaction of AurA with other partner proteins in the context of the centrosome. AurA activity is known to be regulated by several other protein partners, including TPX2, Ajuba, and PP2_B. In cells depleted for HEF1, AurA does not become activated, suggesting the association with HEF1 is functionally important. In cells with HEF1 overexpressed, and able to associate with the centrosome, the stoichiometry of HEF1 is significantly increased, allowing the protein to continue to interact with AurA in spite of phosphorylation by AurA, and thus promoting elevated AurA activity. For both AurA and HEF1, the centrosomal amplification and multipolar spindles seen with overexpression of the proteins is a secondary consequence of cytokinetic failure, with the exact mechanism yet to be defined, but may involve regulation by these proteins of a common effector.

HEF1-depleted cells have abnormally split centrosomes, which accumulate reduced levels of gamma-tubulin in G2^{15,16,26}, have abnormally reduced accumulation of ninein, c-Nap-1, and other proteins, and are deficient in organizing microtubules at mitosis. These defects are likely to be independent of HEF1-AurA signaling, as AurA depletion does not result in centrosomal splitting (Supplemental Figure 5 B, C, D), and may be direct (at the centrosome) or indirect. Our data suggest that HEF1 may normally act to restrain the activity of Nek2^{12,20}, as HEF1 coimmunoprecipitates with Nek2, and Nek2 is hyperactivated in cells with depleted HEF1 or dominant negative HEF1₁₋₄₀₅ (Figure 7 B, C), with increased Nek2 activation previously reported as sufficient to induce splitting. HEF1 may have additional activities required for centrosomal cohesion, as hyperactivation of Nek2 is not sufficient to completely remove c-Nap-1 from centrosomes in interphase cells¹², while accumulation of ninein is an early step in the maturation of daughter centrosomes to mother centrosomes, and has not been described as influenced by Nek2. Such a role for HEF1 is separable from any secondary effect due to defects in cell attachment, as different domains of HEF1 caused splitting versus cell rounding. Intriguingly, recent protein interaction studies of the ancestral HEF1/p130Cas homolog in *Drosophila*²⁷ have suggested this protein associates with a component of the gamma-tubulin ring complex (γ -TuRC), which is important for microtubule nucleation²⁸. It is also interesting that we have previously described the cleavage of HEF1 at amino acid 363 by caspases to produce a p55 species^{3,13}. Although we originally found this p55 species in both mitotic and apoptotic cells, our ongoing work has suggested that the initial idea of HEF1 cleavage at mitosis may have arisen at least in part from contamination of drug-synchronized mitotic populations with apoptotic cells (results not shown). However, the fact that HEF1₁₋₃₅₃ does not associate with centrosomes or AurA in cells, while the slightly larger HEF1₁₋₄₀₅ does, implies that cleavage of HEF1 at this site may have a functional significance in disrupting centrosomal function during cell death.

Definition of HEF1 as a component of the AurA activation machinery is an important finding, providing evidence of a new channel for cross signaling between cell adhesion and mitosis. Further, AurA and Nek2 overexpression and hyperactivation has been observed in many tumors, and is associated with genomic instability²⁹⁻³¹. It was shown for Cas proteins³² that upregulation of p130Cas and/or HEF1 correlates with poor prognosis in breast cancer. It has additionally been shown by us and others that upregulation of Cas proteins influences the

transcription of a number of gene pathways associated with cancer development, enhancing activation of the MAPK pathway and promoting matrix metalloproteinase production (22,³³ and others); it is entirely possible that altered Cas protein levels also induce transcriptional changes that influence genomic stability. Our data imply that beyond their well-defined functions in regulation of susceptibility to apoptosis and cell migration, HEF1 and potentially other members of the Cas family may make additional contributions to the processes of cell transformation through regulation of mitosis.

Methods

Plasmids and constructs

FLAG-, GFP- and GST-fused HEF1 and derivatives were expressed from the vectors pCatch-FLAG, pEGFP-C4¹³ and pGST, respectively. AurA expressed from pCMV-SPORT6-C6 (OpenBiosystems) was used for *in vitro* translation. AurA in pFAST-HT was used for production in baculovirus, and purified by Ni-Sepharose 6FF (Amersham). HEF1-specific and non-specific peptides¹⁴ were expressed from the retroviral vector pUP. Tet-repressible HEF1 in MCF-7 was made using HEF1 in the expression vector pUST-4. Lentiviral constructs were obtained by cloning GFP-HEF1, -HEF1₁₋₃₆₃, or -HEF1₁₋₄₀₅ into pLV-CMV-H4-puro-vector. HEF1 mutants S296A, S296E, S296A/S298A, and S296E/S298E were made using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene); primer sequences available on request.

Cell culture

MCF-7 (breast adenocarcinoma) and HeLa (cervical carcinoma) cell lines were grown in DMEM plus 10% fetal calf serum. The MCF-7-GFP-centrin2 and HeLa-GFP-centrin2 stable cell lines were obtained by transfection of parental cells with pEGFP-centrin2 plasmid³⁴, and G418 selection. Tetracycline-regulated MCF7-tTa-HEF1 stable cell lines were obtained by first infecting the parental MCF-7-tTa cell line (BD Biosciences) with the pUST-4-HEF1 retroviral vector, then selecting with G418/puromycin to produce a mass culture. For analysis of centrosome number in cells that have not undergone mitosis, MCF-tTa-HEF1 cells were plated for 24 – 72 hours +/- tet, with 1 mM hydroxyurea (Sigma). Alternatively, after double thymidine block in the presence of tetracycline, cells were released and grown for 24 and 48 hours in fresh media +/- tet. For growth of cells on poly-L-lysine, laminin, or fibronectin, the procedure described in¹³ was used to prepare coverslips. Cells for analysis were plated on these versus uncoated (normal) coverslips, grown for 48 hours, then centrosomal composition and spreading scored. Examination of centrosomes in non-adherent cells plated on poly-HEMA¹³ was impossible because onset of apoptosis within 24 hours precluded reliable analysis (not shown). For lentiviral infection, pLV- constructs were transfected into the packaging cell line 293-T. After 24 hours, media was collected, filtered through 0.45µm PVDF-filter (Millipore), and applied to MCF-7 cells with polybrene for 2 days, with fresh viral supernatant added every 12 hours. After 48 hours, cells were lysed, analyzed by Western blot analysis, and used for IP-kinase reaction.

Protein expression, Western blotting, and immunoprecipitation

Recombinant proteins were expressed in BL21 (DE3) bacteria, induced with IPTG, and purified using the MicroSpin GST Purification module (Amersham Biotech.). Purified recombinant AurA was purchased from Upstate. For Western blotting and immunoprecipitation (IP), mammalian cells were disrupted by M-PER lysis buffer (Pierce) or NET2 buffer plus protease inhibitor cocktail, and whole cell lysates used either directly for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), or for IP. IP samples were incubated overnight with antibody at 4°C, subsequently incubated for 2 hours with protein A/G-sepharose (Sigma), washed, and resolved by SDS-PAGE. Western blotting was done using standard

procedures and developed by chemoluminescence using the West-Pico system (Pierce). Transiently transfected or infected cells were analyzed for protein expression at 24 – 96 hours post-transfection with Lipofectamine 2000 for plasmids, or Oligofectamine for siRNA (both from Invitrogen). Antibodies used included: rabbit polyclonal antibody to HEF1³, at a 1:100 dilution, mouse monoclonal antibody (mAb) anti-HEF1 14A11 made for this study (1:500), anti- α -tubulin-mouse mAb (Sigma; 1:10,000), mouse mAb anti- γ -tubulin (GTU-88, Sigma; 1:5,000), anti-p130Cas (Transduction Labs, 1:2000), anti-AurA (PharMingen, 1:1,000) for Western, anti-AurA rabbit polyclonal (Abcam ab1287) for IP, anti-Phospho-AurA/T288 (Cell Signaling, 1:1000), anti-HA-antibody (16B12, BabCo, 1:5000), mouse mAb anti- β -actin (AC15, Sigma, 1:10000), mouse mAb anti-cyclin B (GNS-1, BD PharMingen, 1:1000). Rabbit anti-GFP (Abcam ab290) was used for IP, and mouse anti-GFP (JL-8, BD Bioscience) 1:2000 was used for Western blotting. mAb anti-GST (Cell Signaling, 1:2000), rabbit anti-Nek2 (Abcam 1:200) was used for IP. Mouse anti-Nek2 (BD Biosciences, 1:500) and mouse mAb anti-cdc2 antibody (Oncogene, 1:1000) were used for Western blot analysis. Secondary anti-mouse and anti-rabbit HRP conjugated antibodies (Amersham Biotech) were used at a dilution of 1:10000 or 1:20000.

SiRNA

RNA oligonucleotides duplexes (sequences on request) were synthesized targeted to HEF1, Aurora A and to p130Cas, as well as negative controls including scrambled and GFP-directed sequences (Dharmacon, Ambion). After transfection of siRNAs, degree of depletion of target proteins was determined by Western blot.

Cell synchronization

Cells were incubated for 16–18 hours with 2 mM thymidine, washed 2 times in PBS, then either assayed directly (for observation at the G1/S boundary), or returned to fresh medium and allowed to grow for 9–12 hours to observe synchronized progression to mitosis. For synchronization at G2/M boundary, cells were incubated in 1 μ M nocodazole for 14 hours, collected by shake-off, washed in PBS, then either replated in fresh medium on glass cover slips, cultured at 37°C for up to 90 minutes, then fixed for immunofluorescence analysis; or lysed for Western and IP analysis. As an alternative drug-free synchronization approach, an elutriating centrifuge (Beckman J) was used to enrich for G1 or mitotic cell fractions (details on request). For all the synchronization procedures, the predicted cell cycle compartmentalization was confirmed by use of fluorescence-activated cell sorter (FACS) analysis.

Immunofluorescence (IF)

For IF, cells growing on cover slips were fixed with 4% paraformaldehyde, permeabilized, blocked, and incubated with antibodies using standard protocols. Alternatively, to maximize clear signals at centrosomes, cells were fixed in cold methanol (–20°C) for 10 min. blocked and incubated with antibody (see figure legends). Primary antibodies included mouse mAb anti-AuroraA (PharMingen, dilution 1:300), rabbit polyclonal anti-phospho-AuroraA/T288, (Cell Signaling, 1:200), rabbit polyclonal anti-HEF1 1:100, mouse mAb anti-HEF1 (14A11) 1:100, rat mAb anti- α -tubulin (Abcam, 1:200), rabbit polyclonal anti- γ -tubulin (Abcam, 1:200), mouse mAb anti-pericentrin (Transduction Labs, 1:250), mouse mAb anti-phosphohistone 3 (Upstate), rabbit anti-ninein antibody (1:200), mouse mAb anti-c-Nap1 (BD Bioscience, 1:100), and mouse mAb anti-Nek2 (BD Biosciences, 1:100). Secondary antibodies anti-mouse-Alexa-488, anti-rabbit-Alexa488, anti-mouse-Alexa-568, anti-mouse-Alexa-633, anti-rabbit-Alexa 633, and TOTO-3 dye to stain DNA, were from Molecular Probes, Inc. Confocal microscopy was performed using a Radiance 2000 laser scanning confocal microscope (Bio-Rad laboratories, Hercules, CA) coupled to a Nikon Eclipse E800 upright

microscope (Carl Zeiss, Thornwood, NY). Statistical analysis of data by one-way ANOVA was performed using GraphPad InStat 3.0 (San Diego, CA).

Kinase assays

For phosphorylation of HEF1 by AurA, an in vitro kinase assay was performed using bacterially expressed GST-fused HEF1 derivatives. Histone H3 (Upstate) and H1 (Upstate) were used as positive and negative controls for recombinant AurA (Upstate) phosphorylation, using standard methods except as noted in Results. In parallel, aliquots without γ -³²P(ATP) was processed for SDS-PAGE/Coomassie staining (Invitrogen). GST-pulldown assays used wild type AurA translated (pCMV-SPORT6-C6) using TnT Coupled Reticulocyte Lysate System (Promega) mixed with titrated quantities of GST-fused HEF1 derivatives. To analyze HEF1 activation of recombinant, baculovirus-produced AurA, GST-HEF1₁₋₃₆₃ or GST was titrated into a mixture containing recombinant AurA, IPed with anti-AurA, and used for a kinase reaction with γ -³²P(ATP) and histone H3 substrate. Aliquots of the reaction mix were used for SDS-PAGE and Western analysis to confirm levels of AurA; phospho-histone H3 was visualized by autoradiography or by phospho-specific antibody. AurA kinase used in Fig 6C was precipitated from MCF7-tTA-neo or HEF1 cell lines as well as from MCF7 cells treated with control or specific oligonucleotide duplex against HEF1 (siHEF1), using anti AurA antibody (ab1287). The Nek2 kinase assay was performed in standard kinase buffer with addition of an Mg/ATP cocktail (Upstate), with MBP (myelin basic protein) as the substrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HEF1 localization to the centrosome: cell cycle and sequence dependence

A. Cell cycle synchronized populations were analyzed for HEF1 localization to the centrosome and mitotic apparatus. HEF1 is indicated in green, and visualized with α -HEF1-SB-R1 antibodies previously described³. γ -tubulin (red) is used to indicate centrosomes (see arrows). DNA (blue) becomes condensed at mitotic entry. Enlarged views of boxed centrosomes are shown in the bottom right; arrows mark centrosomal location. Images are merged confocal sections. 8 μ m scale bar applies to top row, 6 μ m scale bar applies to all other images. **B.** FACS analysis demonstrating asynchronous MCF7 cells, and MCF7 cells synchronized in G1, S, and G2/M phases, as used for immunofluorescence analysis. **C.** Western analysis of HEF1 levels in the indicated phases of cell cycle, with beta-actin as loading control. The HEF1 doublet represents two phosphorylation-induced isoforms of HEF1: 105kDa and 115kDa (hyperphosphorylated)¹⁰. The broad band migrating at ~95 kD is a non-specific, crossreacting species detected with the rabbit polyclonal antibody, described in¹⁰ (also see Supplemental Figure 1A). **D.** Cells transfected with plasmids expressing GFP-HEF1₁₋₃₆₃ or GFP-HEF1₁₋₄₀₅, and stained with antibody to gamma-tubulin (red). Gray lines in lower panels outline cell periphery. Boxed centrosomes enlarged in insets: arrows indicate other examples. **E.** Fragments of HEF1 analyzed as GFP- or FLAG epitope-tagged fusion proteins, amino acids (a.a.) and degree of localization to the centrosome indicated. The degree was estimated by measurement of signal intensity at the centrosome in the same set of experiments (data not shown). HEF1₁₋₈₃₄ is full length HEF1. *, protein poorly expressed.

Figure 2. Overexpression, stabilization, and depletion of HEF1

A. MCF-7 cells with tetracycline-repressed expression (MCF-7 -tTA) of stably integrated HEF1 or GFP in the presence (+) or absence (-) of tetracycline, measured at 24 or 48 hours after medium change. Western analysis with antibody to HEF1 demonstrates induction following tetracycline removal: the beta-actin protein was used as a loading control. **B.** Western blot analysis of MCF-7 cells infected by retroviruses expressing HA/thioredoxin - tagged peptide fusion proteins (HA-TRX). Levels of HEF1 are stabilized by specific HA-TRX peptides (P1-HEF1, P2-HEF1), but not by non-specific HA-TRX peptides (P1-NS), or by HA-TRX with no peptide inserted (NP). Antibody to HA shows comparable expression of HA-TRX fusions in all lanes. **C.** Western blot analysis of MCF-7 cells treated with siRNAs to HEF1 (siHEF1 and siHEF1a) or a scrambled (Scr) or GFP (siGFP) oligonucleotide duplex shows efficient and specific HEF1 depletion at 48h and 72 h time points. The blot was stripped and re-probed with β -actin as a loading control. All lanes shown were run on single gel; white lines here and in following figures indicate excision of intervening bands.

Figure 3. Overexpression and stabilization of HEF1 induce supernumerary centrosomes and multipolar mitotic spindles

A. MCF-7 cells with tet-repressed HEF1 (tTA-HEF1) were uninduced (top row) or induced by tetracycline removal (bottom 3 rows). Cells were treated to visualize DNA (blue) alpha-tubulin (green) and gamma-tubulin (red) for immunofluorescence; representative mitoses are shown. Scale bar for images in this panel is 4 μ M. **B.** Quantitation of multipolar spindles scored in cells with induced (–Tet) or un-induced (+Tet) HEF1 or GFP expression, or treatment with peptides stabilizing endogenous HEF1: specific for HEF1 (H), non-specific (NS), or peptide negative control (NP) HA-TRX fusions. The histogram indicates the percentage of cells with multipolar spindles under different conditions. Red bars indicate conditions with increased HEF1 levels. 3 independent experiments were performed, resulting in the assessment of 150 mitoses in total for each condition. ***, $P < 0.001$ versus negative control condition. **C.** tTA-HEF1 cells were first synchronized in double thymidine blocked, then released into medium with tetracycline (blue, HEF1 repressed) or without tetracycline (red, HEF1 induced), and the number of centrosomes scored at 0, 24, or 48 hours. 150 cells were counted in 3 experiments. ***, $P < 0.001$ difference minus versus plus tetracycline for each time point; difference at 0 hours is not significant. **D.** tTA-HEF1 cells were presynchronized in thymidine, then placed in medium with hydroxyurea, and with or without tetracycline, and centrosomes scored up to 72 hours. 100 cells were counted in 3 experiments. No significant differences were seen.

Figure 4. Depletion of HEF1 induces centrosomal splitting and a monoastrial mitotic spindle

A. MCF-7 cells were transfected with either a control scrambled (Scr, top panels) siRNA, or an siRNA specific for HEF1 (siHEF1; center and bottom panels) for 48 hours, then processed for immunofluorescence using markers to gamma-tubulin (red), alpha-tubulin (green), and DNA (blue). Shown, representative mitoses. Scale bar for images in this panel is 5 μ m. **B.** Immunofluorescence analysis of MCF-7 cells with stably expressed GFP-centrin2 (green) 48 hours post-transfection of scrambled (Scr) or HEF1-directed (siHEF1) siRNAs was used to calculate the frequency of split (top panel) versus closely paired (bottom panel, Scr) centrioles (indicated by arrows, with boxed examples shown in insets). The centrioles were considered as split if the distance between them was 2 μ m or more¹². Results shown are for depletion

with siHEF1; comparable phenotypes are seen with siHEF1a. DNA is shown in blue. Scale bar for images in this panel is 5 μ M. **C.** For quantification, >150 cells were counted from each of at least 3 separate experiments for HEF1-depleted (siHEF1) or control (Scr) siRNA treated cells, and 2 experiments for si p130 and siGFP. All counting was done at a time point after 48h of siRNA treatment. ***, $P < 0.001$ for siHEF1 versus either Scr or siGFP; */**, $P < 0.01$ versus Scr and $P < 0.05$ versus siGFP. **D.** FACS analysis of cells depleted with indicated siRNAs at 48 hours post-transfection.

Figure 5. HEF1 associates with AurA and control AurA activation

A. MCF-7 cells were depleted with HEF1-directed siRNA (siHEF1) or a scrambled control (Scr). Mitotic cells stained with antibodies directed at AurA (green), phospho-AurA/T288 (P-AurA, red), and DNA (blue) are shown. Scale bar (5 μ m) applies to all panels. **B.** MCF-7 cells were transfected with scrambled control (Scr) or HEF1-directed (siHEF1) siRNA for 48 hours, then collected and part of the sample elutriated. Non-elutriated (Asyn.), G1/S, or G2-M enriched populations were used for Western blot analysis using the antibodies indicated (left). White vertical lines indicate where intervening (irrelevant) lanes run on the gel have been excised from image. **C.** MCF-7 cells prepared as in **B** were used for co-immunoprecipitation. Top, immunoprecipitation with control IgG or Cas antibody (cross-reactive with p130Cas and

HEF1; efficient direct precipitation of both proteins), and Western blot using antibodies as indicated to left. Bottom, immunoprecipitation with control IgG or AurA antibody, and Western blot as indicated. For each experiment, co-immunoprecipitation is compared in cells depleted for HEF1, or treated with scrambled siRNA, to confirm specific requirement of HEF1 in co-immunoprecipitation.

Figure 6. Delineation of the HEF1-AurA interaction

A. In vitro translated ³⁵S-labelled AurA was used for pulldowns with GST-fused fragments of HEF1 or GST. Autoradiography shows AurA (top panel) and Coomassie blue (CB) shows GST-fusions (bottom panel). In lane “R”, 20% of a total AurA ³⁵S-labelled reaction mixture is shown: for each pulldown, a complete reaction mixture is used. **B.** Decreasing quantities (4, 2, or 1 μg) of GST-fused HEF1₁₋₃₆₃ or GST were used in an *in vitro* kinase reaction with recombinant AurA. Reactions were visualized with antibody to AurA or phospho-AurA/T288, or with CB to show GST-fusions. Phosphorylated histone H3 was visualized by autoradiography. **C.** AurA was immunoprecipitated from MCF7 cells that were tetracycline repressible for vector (tTA) or HEF1 (tTA-HEF1) expression, + or –tet; and additionally, from cells treated with siRNA to HEF1 (siHEF1) or to scrambled control (Scr). The

immunoprecipitated AurA was incubated with recombinant histone H3 with γ - ^{32}P -ATP in an in vitro kinase assay, resolved by SDS-PAGE, and visualized by antibody to AurA, Coomassie blue (CB) staining to detect H3, and autoradiography. **D.** GST, GST-fusion proteins, or histone H1 or H3 (CB, left) were incubated with AurA and γ -ATP- ^{32}P in vitro. Right, autoradiograph of phosphorylated proteins. **E.** GST- HEF1₁₋₃₆₃ wild type (wt), or with ala or glu mutations [S296A (A), S296A/S298A (AA), S296E (E), S296E/S298E (EE)] were incubated with AurA and γ -ATP- ^{32}P in vitro. Top, CB shows GST-HEF1 fusions; Bottom, autoradiograph of phosphorylated GST-fusions. **F.** The GST-fused derivatives of HEF1 described in **E** were mixed with recombinant AurA and histone H3 (H3) in the presence of γ -ATP- ^{32}P , incubated, and level of histone-H3 phosphorylation determined by autoradiography. Top, CB shows GST-fusions; second row, input histone H3; third row, phospho-histone H3. After incubation, immunoprecipitated AurA (IP:AurA) was probed with antibody to AurA and GST. **G.** GFP-fused full length HEF1 (wt or with mutations described in **E**) were transfected into MCF7 cells. After 24 hours, sequential western blot analysis of immunoprecipitated AurA was done using anti-AurA and anti-GFP (top and middle panels). Bottom, anti-GFP shows expression of GFP-fusions in total lysate; bands at ~70–80 kD are degradation products.

Figure 7. HEF1 depletion affects Nek2 activation and association of proteins with the PCM
A MCF7 cells with integrated GFP-centrin were treated with scrambled control siRNA (Scr), or siRNA to HEF1 (siHEF1), and stained for immunofluorescence with antibodies to HEF1, c-Nap1 or Nek2, as indicated. Scale bars equal 10 μ m. **B**. MCF7 cells were either treated with siRNAs (left panel), or infected with retrovirus expressing full length HEF1 or HEF1 truncations, as for Supplemental Figure 6C. Nek2 kinase was immunoprecipitated, and used for in vitro kinase assays with myelin basic protein (MBP) as a substrate. A comparable level of HEF1 or AurA protein depletion (Supplemental Fig. 5A), and similar levels of overexpression of HEF1 and truncations (not shown) was confirmed with antibodies to GFP and/or HEF1 and AurA, in whole cell lysates. **C**. Antibody to Nek2 or Cdc2 was used for immunoprecipitation from asynchronous MCF7 cells. Immunoprecipitates were probed with antibodies to HEF1, Nek2, or Cdc2, as indicated; top, middle, and bottom panels.

Figure 8. HEF1 activities at the centrosome and in cell spreading

For histograms *** represents $P < 0.001$, ** represents $P < 0.01$, and * represents $P < 0.05$, in reference to the GFP control. **A.** Centrosomal splitting in GFP-positive cells transfected with HEF1 truncations. Top, MCF7 cells; bottom, HeLa cells. Bars indicate split centrosomes. Three (MCF7) and two (Hela) independent experiments were performed ($n = 100$ cells/experiment). **B.** Cell spreading in GFP-positive cells transfected with HEF1 truncations. Top, MCF7 cells; bottom, HeLa cells. 100 cells were counted in each of 3 experiments. Cell spreading analysis was performed as described in ¹³, with calculation of area based on pixels within a traced cell perimeter. IF with α -paxillin was used to confirm increased size and formation of focal adhesions in cells plated on laminin and fibronectin. **C.** Western analysis of expression of different HEF1 truncations expressed as GFP-fused proteins in MCF7 cells. Blot probed with antibody to GFP. Lanes are GFP-fusions to 1, 654-834; 2, 351-363; 3, 1-363; 4, FL; 5, 1-405. **D.** Mitotic spindle defects induced by HEF1 overexpression in cells plated on different

matrices. Tet-repressible MCF-7 derived cells were grown in the presence (+) or absence (–) of tet 48 hours after plating on glass coverslips (–), or coverslips coated with poly-L-lysine (PLL), fibronectin (FN), or laminin (LAM). Bars, multipolar or malformed spindles. 100 mitotic cells were counted in each of 3 independent experiments. **E, F.** Centrosomal splitting induced by depletion of HEF1 (siHEF1), or overexpression of dominant negative HEF1 (GFP-fused HEF1₁₋₄₀₅), in cells plated on different matrices. For **E**, MCF7 cells with integrated GFP-centrin2 were transfected with siRNA containing scrambled sequence (Scr) or targeted to HEF1 (siHEF1). For **F**, MCF7 cells were transfected with GFP-fused HEF1₁₋₃₆₃ or HEF1₁₋₄₀₅, and centrosomes visualized by antibody to gamma-tubulin. Following treatment, cells were plated on glass coverslips (–), or coverslips coated with poly-L-lysine (PLL), fibronectin (FN), or laminin (LAM). Bars, split centrosomes. 100 cells were counted in each of 3 experiments. For **D–F**, the differences induced by matrix were not statistically significant, except *, $P < 0.05$ for PLL in **D**.