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ARTICLES

Nucleolar release of Hand1 acts as a molecular switch to determine cell fate

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The bHLH transcription factor Hand1 is essential for placentation and cardiac morphogenesis in the developing embryo. Here we implicate Hand1 as a molecular switch that determines whether a trophoblast stem cell continues to proliferate or commits to differentiation. We identify a novel interaction of Hand1 with a protein that contains an I-mfa (inhibitor of myogenic factor) domain that anchors Hand1 in the nucleolus where it negatively regulates Hand1 activity. In the trophoblast stem-cell line Rcho-1, nucleolar sequestration of Hand1 accompanies sustained cell proliferation and renewal, whereas release of Hand1 into the nucleus leads to its activation, thus committing cells to a differentiated giant-cell fate. Site-specific phosphorylation is required for nucleolar release of Hand1, for its dimerization and biological function, and this is mediated by the non-canonical polo-like kinase Plk4 (Sak). Sak is co-expressed in Rcho-1 cells, localizes to the nucleolus during G2 and phosphorylates Hand1 as a requirement for trophoblast stem-cell commitment to a giant-cell fate. This study defines a novel cellular mechanism for regulating Hand1 that is a crucial step in the stem-cell differentiation pathway.

Understanding how regulatory factors control differentiation of stem- or progenitor-cell populations in a specific lineage would help to reveal how cell fate is established and maintained and the mechanisms that restrict cell potency.

The trophoblast cell lineage is a model system for the study of cell fate. In mice there are only four major differentiated cell types that can be derived from trophoblast stem (TS) cells: trophoblast giant cells (TG), spongiotrophoblast (SpT), syncytiotrophoblast (SynT) and glycogen trophoblast (GlyT) cells[1,2]. TG cells are large polyploid cells that mediate invasion into the uterus and promote local and systemic adaptations in the mother that are necessary for embryonic growth and survival[3]. The differentiation and/or maintenance of TG cells requires the bHLH factor Hand1 (refs 4–7).

To explore the molecular basis for Hand1 promotion of cell-cycle exit and restriction of trophoblast stem cells to a giant-cell fate, we sought to identify novel modes of Hand1 regulation. Unlike most bHLH proteins, Hand1 exhibits promiscuous dimerization properties[8,9]. We reasoned that the broad array of Hand1 interactions, with both class A and class B bHLH proteins, might extend to non-bHLH factors, which in turn may be responsible for mediating Hand1 biological activity.

RESULTS

Hand1 interacts with the murine orthologue of HICp40

In a yeast two-hybrid (Y2H) screen with Hand1 as bait, we isolated a number of Hand1-interacting factors from a murine embryonic day (E) 9.5/E10.5 cDNA library[10]. Sequence analysis of one such factor revealed an 89% nucleotide identity and a 95% amino-acid identity with HIC protein (human I-mfa-domain-containing protein; Fig. 1a). The region of identity mapped to the cysteine-rich carboxy-terminal I-mfa domain was 74% identical to the corresponding region of the cellular protein I-mfa. The interaction of Hand1 with HIC was confirmed by GST pulldown and coimmunoprecipitation assays (Fig. 1b). HIC consists of two isoforms of molecular weights 32 kDa and 40 kDa (denoted HICp32 and HICp40) arising from two different translation initiator codons[11]. The mouse orthologue of HIC, murine I-mfa-domain-containing protein (MIC), is represented in the EST database (GenBank accession number BB222360) and includes a coding region with 81% amino-acid identity to that of HICp32. The full-length *MIC* gene can be located in ENSEMBLE (NM_175088), where transcript information predicts an exon–intron structure and a further 5′ coding sequence with homology to the longer human isoform HICp40.

HICp40 inhibits Hand1 through nucleolar sequestration

I-mfa is a myogenic repressor that directly inhibits the bHLH factors MyoD and Myf5 (ref. 5). I-mfa also inhibits transactivation by the bHLH factor Mash2 during trophoblast differentiation[13] and interacts with Hand1 but does not inhibit Hand1 activity[13]. HIC proteins also function as widespread transcriptional repressors, inhibiting activation

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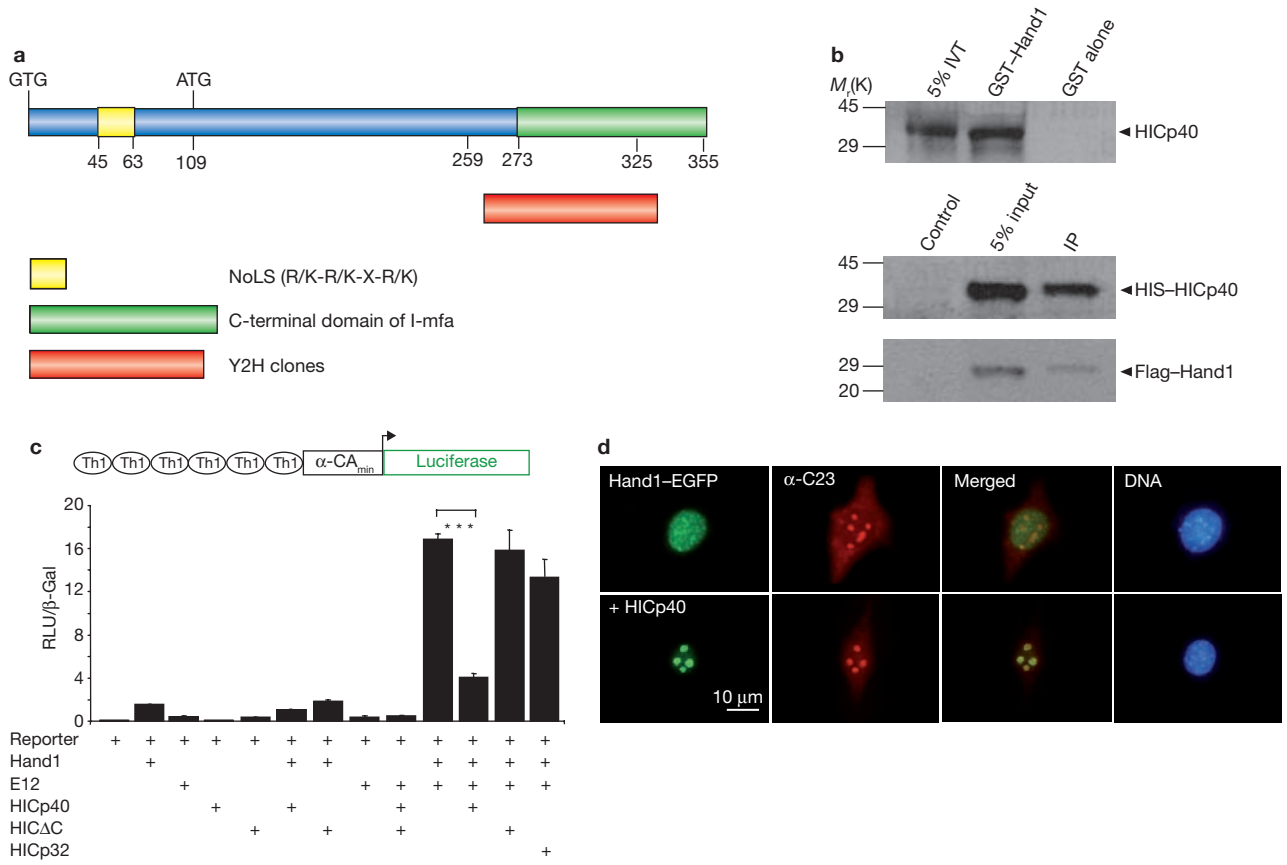

Figure 1 HICp40 specifically interacts with Hand1 and negatively regulates its transcriptional activity through nucleolar sequestration. (**a**) The location of the Hand1-interacting clones identified by yeast two-hybrid (Y2H) in the HICp40 protein sequence is indicated (red box). The yellow region represents the nucleolar localization sequence (NoLS), and the green region represents the C-terminal I-mfa domain of HICp40. (**b**) Confirmation of Hand1 and HICp40 interaction by pulldown assay with GST–Hand1 fusion protein and *in-vitro*-translated HICp40 and coimmunoprecipitation of Flag–Hand1 with HIS–HICp40 in transfected NIH3T3 cells with polyclonal α-HIS antibody. (**c**) HICp40 negatively regulates Hand1 transcriptional activity in a transient transfection reporter assay with the E-factor E12 and a mock Hand1 target gene comprising six high-affinity Hand1-binding sites (Thing 1 boxes; Th1)[10] sequences upstream of a minimal α-cardiac actin promoter and a luciferase cassette[44], and this is dependent on both the NoLS and I-mfa domain. (**d**) Hand1–EGFP is predominantly localized throughout the nucleus in transfected NIH3T3 cells with a small percentage of cells (20%) exhibiting weak staining in the nucleolus as confirmed by immunostaining for the nucleolus-specific protein nucleolin (C23), whereas ectopic HICp40 causes Hand1–EGFP to become predominantly (>90%) expressed in the nucleolus; measurements in **c** are mean±s.e.m (n=3), ***P<0.001; the scale bar represents 10 μm. Full scans for **b** are presented in Fig. S4.

of the HIV-1 long terminal repeat (LTR) by sequestering the regulatory protein HIV-1 Tat in the cytoplasm[14] and preventing DNA binding of the HMG box transcription factor Tcf3 to block Wnt signalling[15]. Because HIC and I-mfa proteins function as negative modulators of different pathways, we sought to assess the functional significance of the HIC–Hand1 interaction. HIC significantly reduced reporter activity mediated by Hand1–E-factor (E12) (Fig. 1c), and this repression was restricted to the p40 isoform and dependent on the C-terminal I-mfa domain (Fig. 1c). HICp40 is localized almost exclusively to the nucleolus[11,16,17], and, therefore, we investigated whether nucleolar sequestration of Hand1, to restrict its access to target genes, might be responsible for inhibiting Hand1 activity. Compartmentalization of transcription-associated proteins within nuclear bodies has been suggested to act as an important mechanism for regulating gene expression[18]. For example, the activity of p53 is regulated by the nucleolar sequestration of its repressor MDM2 (refs 19–22) and c-Myc-induced progression through the cell cycle is inhibited by the sequestration of the protein in the nucleolus[23]. Transfection of NIH3T3 cells with Hand1 tagged with enhanced green fluorescence protein (EGFP) in the presence of HICp40 restricted the otherwise predominant nuclear wide localization of the fusion protein to the nucleolar compartment (>90% of transfected cells exhibited nucleolar fluorescence) as confirmed by colocalization with anti-nucleolin (α-C23; Fig. 1d).

Nucleolar release of Hand1 underpins trophoblast stem-cell differentiation

To assess the physiological significance of nucleolar sequestration of Hand1 *in vivo* we took advantage of the rat choriocarcinoma-1 (Rcho-1) model system. Rcho-1 cells are a faithful model of *in vivo* TS cells[3,24,25], and they can be induced to exit the mitotic cell cycle and undergo endoreduplication concomitant with differentiation into TG cells[26]. Hand1 has been functionally implicated in the differentiation of Rcho-1 cells to form TG cells, but the detailed mechanism is not known[4,8,13].

In proliferating Rcho-1 stem cells, endogenous Hand1 is immunolocalized almost exclusively to the nucleolus (Fig. 2a). Rcho-1 cells were induced to differentiate through a change in serum conditions from 20% fetal bovine serum (FBS) to 10% horse serum as previously described[26] (see Methods). During differentiation, Hand1 exits the nucleolus and

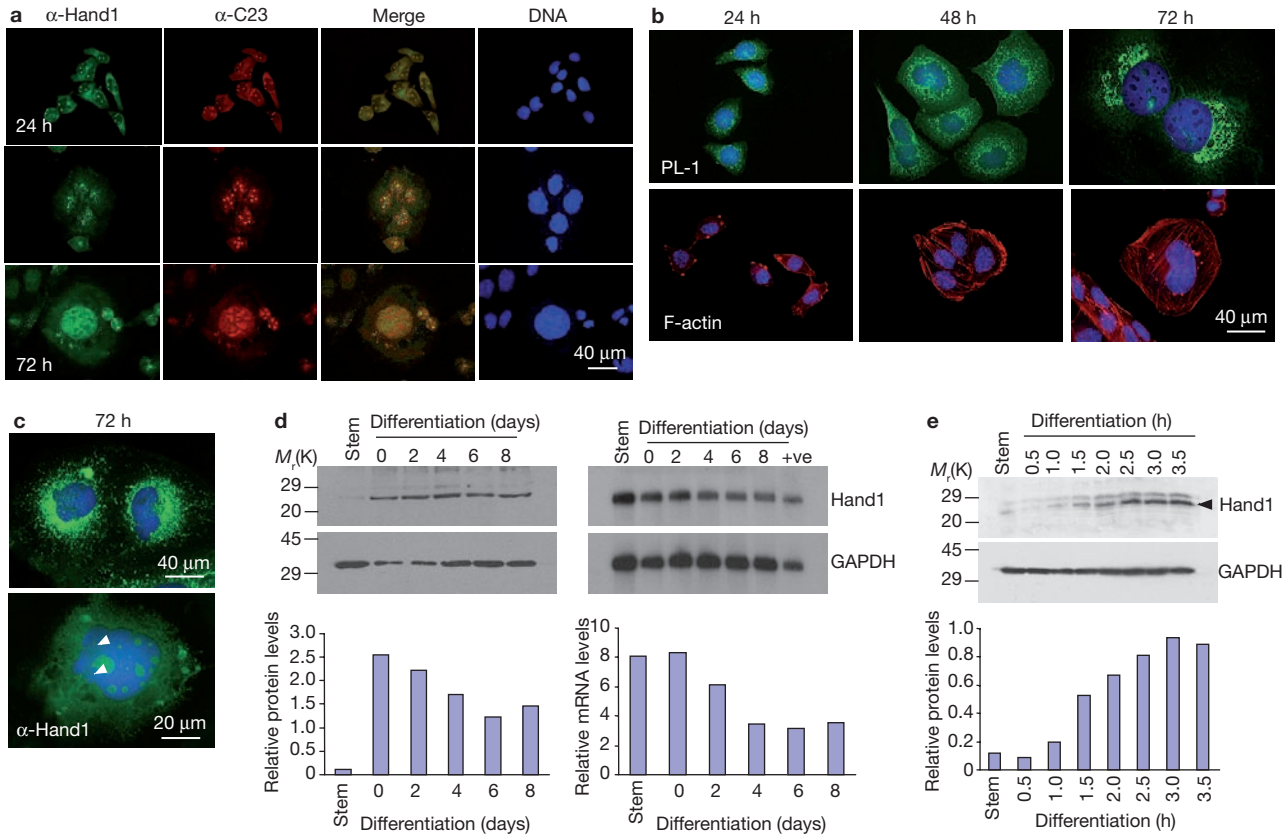

Figure 2 Nucleolar release of Hand1 coincides with Rcho-1 stem-cell commitment to a trophoblast giant-cell fate. (**a**) Immunostaining for α-Hand1 and α-C23 over a 72-h time course of Rcho-1 differentiation reveals that endogenous Hand1 is localized to nucleoli in Rcho-1 stem cells but is released rapidly into the nucleus coincident with a commitment to differentiation. (**b**) Rcho-1 giant cells induced by serum withdrawal (20% FBS to 10% horse serum (HS)) undergo a marked upregulation in the giant-cell-specific marker PL-1 and a complex cytoskeletal rearrangement, as indicated by phalloidin staining for F-actin. (**c**) Immunostaining for α-Hand1 over a 72-h time course of Rcho-1 differentiation reveals that in a few TG cells (<5%) endogenous Hand1 is localized to the perinuclear region (upper panel, see **a**) or occasionally retained in the nucleolus (lower panel); however, a proportion of Hand1 in both instances is translocated nuclear-wide (highlighted by white arrowheads) and seems sufficient to induce TG-cell differentiation. (**d**) Western (lysates subtracted for the nucleolar fraction) and northern analyses for Hand1 protein and mRNA expression, respectively, in Rcho-1 cells over a time course of differentiation in 10% horse serum (HS; stem, 0–8 days). (**e**) Elevated protein expression of Hand1 at the onset of differentiation (day 0; 3-h exposure to HS) is not accompanied by a corresponding elevation in Hand1 transcript levels and is caused by nucleolar–nuclear redistribution of Hand1. Elevated protein expression of Hand1 in the nucleus is observed at the onset of differentiation after 1-h exposure to HS (stem, 0–3.5 h), owing to nucleolar–nuclear redistribution of Hand1. Full scans for **d** and **e** are presented in Fig. S4. Scale bars in **a, b** and **c** (upper) represent 20 μm, and the scale bar in **c** (lower) represents 20 μm.

becomes increasingly nuclear (Fig. 2a). Ultimately, diffuse, nuclear wide Hand1 is observed in *bona fide* giant cells as determined by a characteristic upregulation in PL-1 (placental lactogen 1) and cytoskeletal reorganization[26,27] (Fig. 2b). Cohorts (<5%) of TG cells exhibiting nucleolar or perinuclear Hand1 were observed (Fig. 2c) but in both cases a fraction of the total Hand1 was also localized in the nucleus, suggesting that any amount of endogenous Hand1 translocation may be sufficient to drive trophoblast differentiation. Western-blot analysis, on nuclear preparations (subtracted for nucleolar content), taken over a time course of Rcho-1 differentiation confirmed the absence of Hand1 in stem cells (Fig. 2d). Notably, translocation of Hand1 to the nucleus becomes evident 3 h after serum withdrawal (Fig. 2d) and is initiated after only 1 h (Fig. 2e). Northern-blot analysis on the equivalent time course of Rcho-1 differentiation revealed no change in Hand1 message at the onset of stem-cell differentiation (Fig. 2d), confirming the upregulation in nuclear Hand1 protein levels was caused by change in Hand1 protein subcellular localization.

We next investigated whether ectopic expression of a Hand1–EGFP fusion protein in Rcho-1 stem cells, under non-differentiating conditions, promotes commitment to a giant-cell fate and tracked the dynamic change in subcellular localization of EGFP fluorescence over time. In stem cells, 24-h post transfection, Hand1–EGFP was extensively localized to nucleoli (Fig. 3a). Further observations, recorded at 48 h and 72 h, revealed Hand1–EGFP dispersal from the nucleoli in cells committed to differentiate (Fig. 3a). Hand1–EGFP distribution became increasingly nuclear wide, which was coincident with adoption of a TG-cell fate (Fig. 3a, b). Hand1–EGFP-induced TG cells were stained for PL-1 and phalloidin to confirm a *bona fide* giant-cell phenotype (Fig. 3b). The relative proportions of transfected cells with nucleolar versus nuclear-wide EGFP fluorescence and TG cells were determined at each time point, and they revealed significant increases in both nuclear-wide expression of Hand1–EGFP and TG cells over time (Fig. 3c). The fact that the level of nuclear translocation of Hand1–EGFP does not correlate precisely with the increase in giant-cell

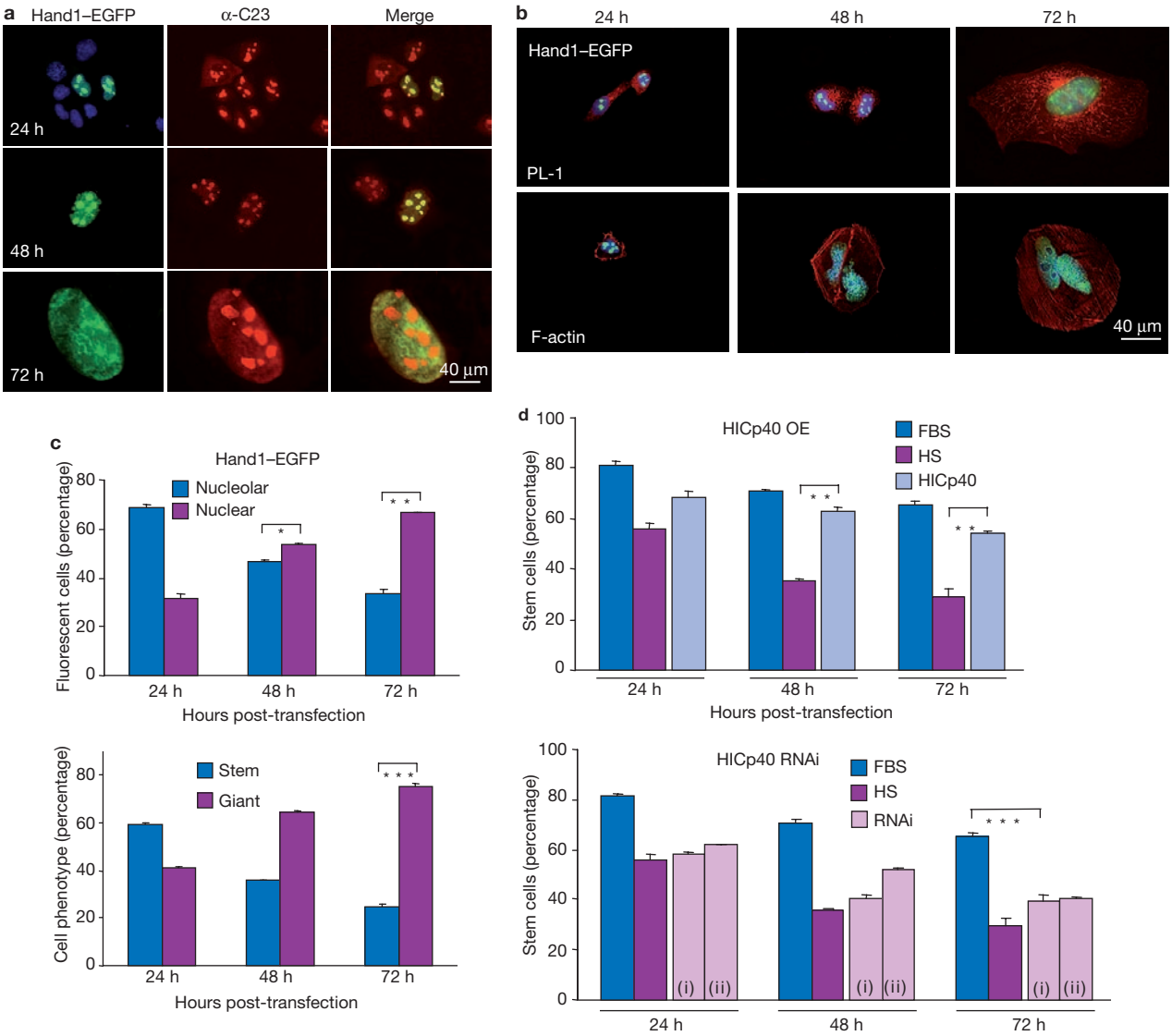

Figure 3 Hand1–EGFP-induced differentiation of Rcho-1 cells produces *bona fide* trophoblast giant cells, and nucleolar release of Hand1 takes place along with commitment to a giant-cell fate. (**a**) In non-differentiating medium (20% FBS), Hand1–EGFP initially localizes exclusively to the nucleoli of Rcho-1 stem cells but assumes a more nuclear-wide distribution inducing differentiation towards a giant cell (TG-cell) fate. (**b**) Rcho-1 cells induced to differentiate by Hand1–EGFP (in 20% FBS) produce giant cells with equivalent PL-1 upregulation and cytoskeletal rearrangement. (**c**) Cell counts of Hand1–EGFP-expressing Rcho-1 cells reveals a significant reduction in nucleolus-localized fusion protein and a corresponding elevation in nucleus-wide localization over 72 h, which is coincident with a reduction in number of stem cells and increased number of TG cells. (**d**) Overexpression of HICp40 in Rcho-1 cells cultured in horse serum significantly inhibits TG-cell differentiation, whereas knockdown of HICp40 in non-differentiating conditions (indicated by FBS) by two independent RNAi sequences significantly promotes TG-cell differentiation; measurements (*n* is the number of cells counted per treatment at each time point) in (**c**; *n*=450) and (**d**; *n*=350) are mean±s.e.m, $*P<0.05$, $**P<0.01$, $***P<0.001$; scale bars represent 40 μm.

differentiation (Fig. 3c) suggests, as observed for endogenous Hand1 (see Fig. 2c), that the release of a proportion of the total Hand1–EGFP is sufficient to commit the majority of stem cells to differentiate. The kinetics of the change in Hand1–EGFP localization were recorded using time-lapse video microscopy, which revealed the release of Hand1–EGFP from the nucleolus over a 12-h time period as individual transfected cells began to differentiate, which was coincident with decreased motility and an expansion in size (see Supplementary Movie).

Nucleolar localization of Hand1–EGFP was also observed in primary murine TS cells[28] (see Supplementary Fig. S1a), which supports the authenticity of the Rcho-1 model with respect to the trophoblast *in vivo* and rules out any cell-line-dependent effects. Moreover, this localization pattern was confirmed as specific to Hand1 because EGFP fusions with the closely related Hand2 and MyoD did not localize to the nucleolus (see Supplementary Fig. S1b, c).

We next assessed the effects of a gain and loss of HICp40 function on Rcho-1 cell differentiation. Overexpression of HICp40 (HICp40–EGFP) significantly inhibited Rcho-1 cell differentiation (Fig. 3d), whereas in non-differentiating conditions, RNA interference (RNAi)-mediated knockdown of HICp40 using two independent RNAi sequences (see Supplementary Information, Fig. S2 for levels of HICp40 knockdown) significantly promoted Rcho-1 cell differentiation (Fig. 3d). Gain in HICp40 function was associated with elevated numbers of stem cells and a significant increase in nucleolar Hand1

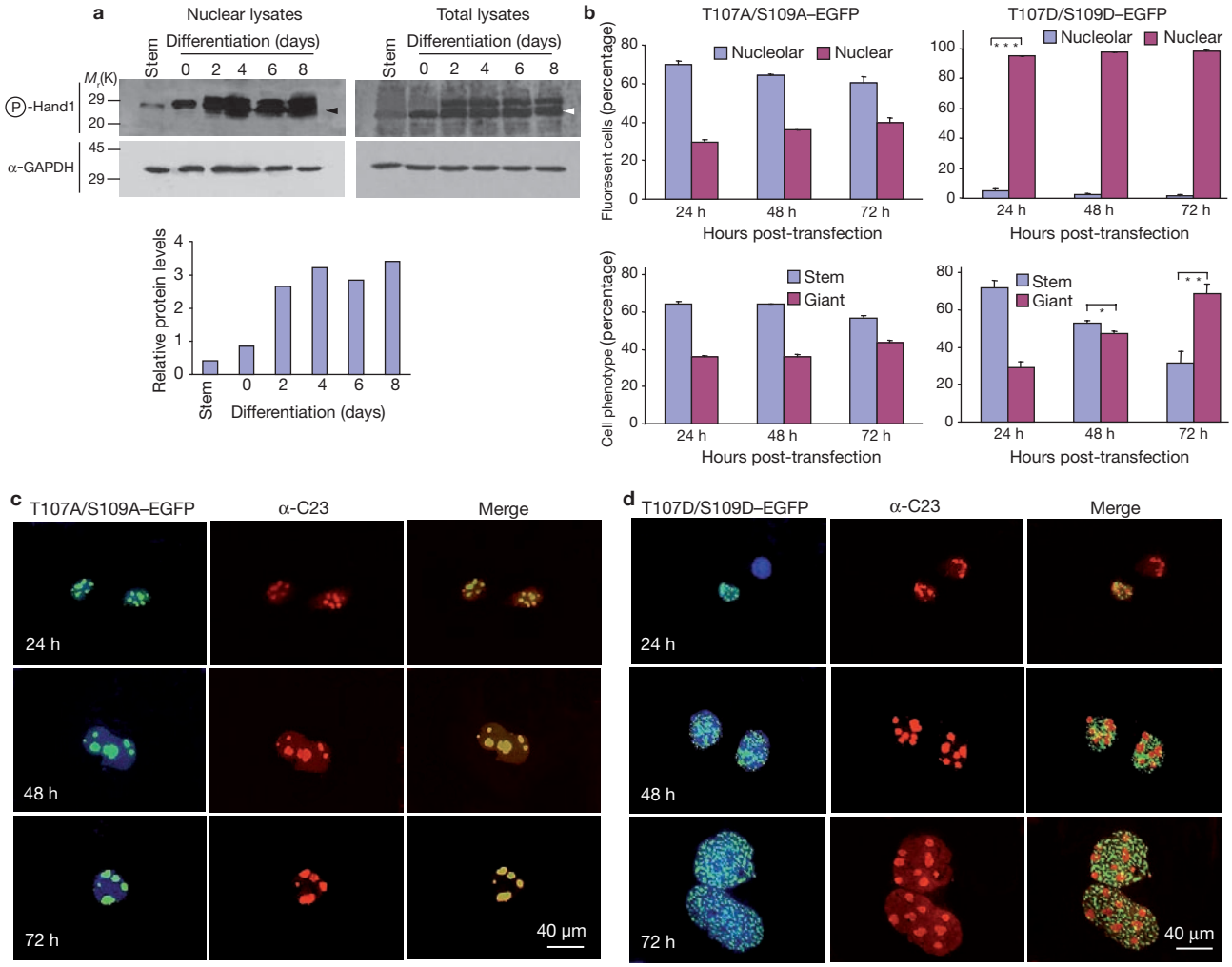

Figure 4 Phosphorylation of threonine (T107) and serine (S109) residues in helix 1 underlies Hand1 nucleolar release during Rcho-1 giant-cell differentiation. (**a**) Phosphoserine western (lysates subtracted for the nucleolar fraction, Nuclear, and total protein lysates, Total) on Rcho-1 cells over a time course of differentiation in 10% horse serum (HS; stem, 0–8 days). Elevated nuclear, phosphorylated Hand1 at the onset of differentiation (day 0; 3-h exposure to HS) suggests appropriate nucleolar–nuclear redistribution of active phosphorylated Hand1. (**b**) Mutation of T107 and S109 to alanines prevents phosphorylation of Hand1 and results in a failure of Hand1–EGFP to exit the nucleolus over a 72-h time course, (**c**) along with a corresponding failure to induce stem cells to differentiate into TG cells. (**d**) Mutation of T107 and S109 to aspartic acid produces a 'phosphorylation mimic' that either prevents Hand1–EGFP entry/sequestration into the nucleolus or facilitates its immediate release upon entry, revealing a distinct punctate localization throughout the nucleus, consistent with the ability of this T107D/S109D mutant to induce TG-cell differentiation albeit with a lower efficiency compared with wild-type Hand1–EGFP (see Fig. 2d). (**b**) Measurements: n= 400 are mean±s.e.m, $*P<0.05$; $**P<0.01$; $***P<0.001$; scale bars represent 40 μm. Full scans for **a** are presented in Fig. S4.

(Fig. 3d; control 43.9±1.87 (mean±s.e.m.) versus overexpression 77.6±2.67 (mean±s.e.m.) for nucleolar Hand1 localization from three replicates at 72 h; P=<0.001), whereas a reduction in HICp40 resulted in elevated numbers of giant cells and increased nuclear wide Hand1 (Fig. 3d; the mean percentage of cells with nuclear wide Hand1±s.e.m. is 28.6%±2.08 (control) versus RNAi (i) 47.1±1.93 and RNAi (ii) 46.4±2.39; from three replicates at 72 h, P=<0.001). These findings are consistent with a role for HICp40-mediated nucleolar sequestration and release of Hand1 during trophoblast differentiation.

Phosphorylation of Hand1 underlies its nucleolar–nuclear translocation

We next investigated the mechanism underlying the release of Hand1 from the nucleolus. Isolated studies in budding yeast have revealed that nucleolar release of the protein phosphatase Cdc14 is dependent on phosphorylation by the polo kinase Cdc5, and this is as an essential step towards exit from mitosis[29]. In a previous study, phosphorylation of Hand1 was shown to increase during Rcho-1 cell differentiation. Specifically, threonine T107 and serine S109 residues, located in helix 1 of the bHLH domain, are phosphorylated *in vivo* to determine the dimerization properties of Hand1 as an important step in the differentiation programme[30]. We reasoned, therefore, that phosphorylation of Hand1 might underlie its nucleolar release as a prerequisite for heterodimer formation and biological activity in the nucleus. Phosphoserine western analysis to detect levels of phosphorylated Hand1 in nuclear preparations (subtracted from nucleolar content) taken over a time course of Rcho-1 differentiation revealed a significant increase in nuclear-phosphorylated Hand1 at the onset of, and persisting throughout, trophoblast differentiation (Fig. 4a).

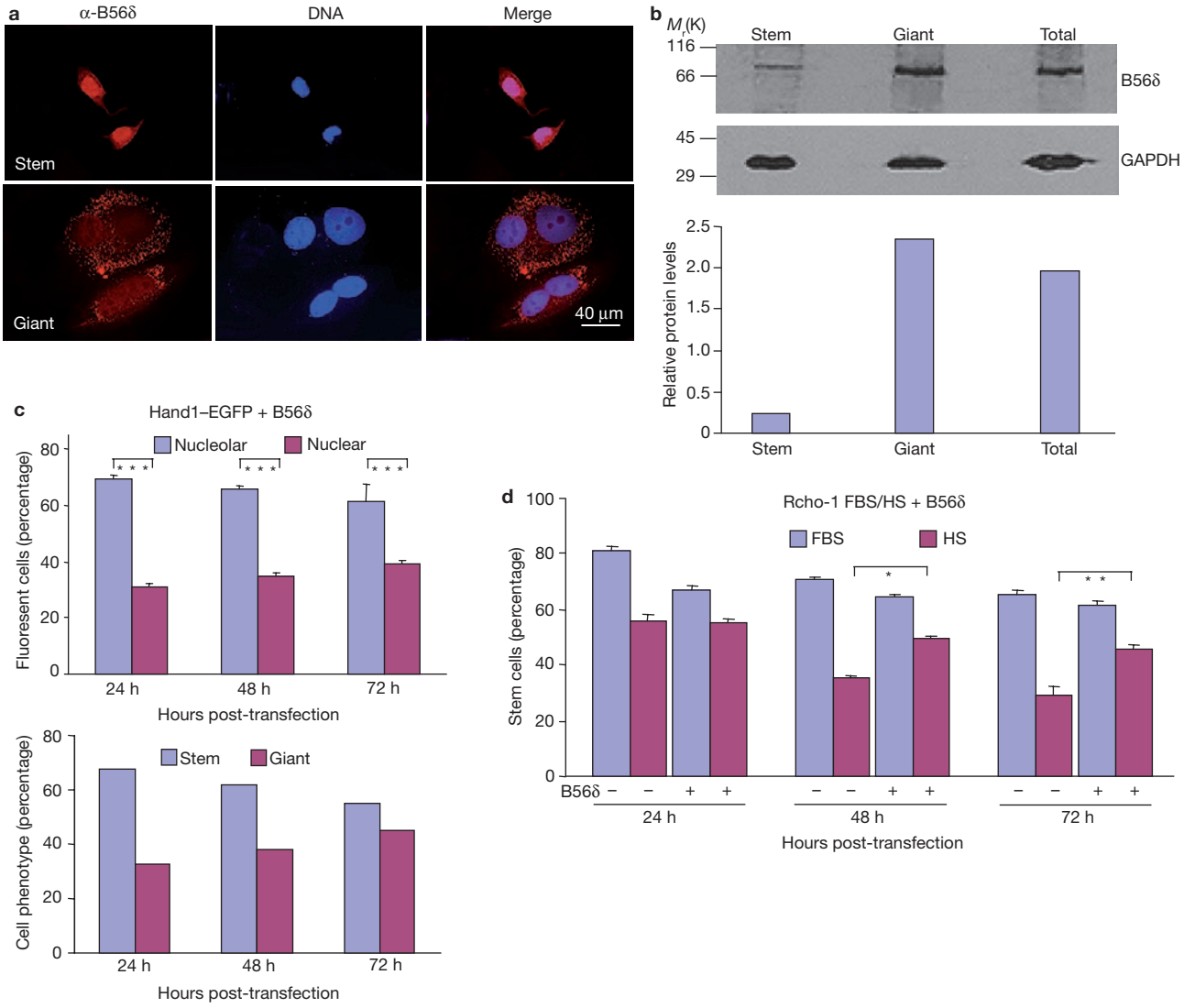

Figure 5 The B56δ phosphatase subunit undergoes nucleolar/nuclear redistribution to the cytoplasm during Rcho-1 differentiation and inhibits both Hand1–EGFP- and horse-serum-induced differentiation. (**a**) B56δ is localized throughout the nucleus, including in the nucleoli in Rcho-1 stem cells but relocates to the cytoplasm upon terminal TG-cell differentiation. (**b**) Western analysis on Rcho-1 lysates, subtracted for the nucleolar fraction, reveals an elevated nuclear localization of B56δ and subsequent decrease in nucleolar localization during stem-cell differentiation before cytoplasmic redistribution in definitive giant cells. (**c**) Ectopic expression of B56δ prevents nucleolar release of Hand1–EGFP and blocks TG-cell differentiation, and (**d**) when expressed 24 h after serum replacement (and thus is in differentiating conditions) also significantly inhibits horse-serum-induced TG-cell differentiation; measurements (**c**; n=330) and (**d**; n=350) are mean±s.e.m, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; scale bars represent 40 μm. Full scans for **b** are presented in Fig. S4.

To address whether phosphorylation of Hand1 at T107 and/or S109 could bring about release of Hand1 from the nucleolus during Rcho-1 stem-cell commitment, we expressed mutant Hand1–EGFP fusion proteins in which T107 and S109 were either substituted for alanine or aspartate residues (T107/S109A or T107/S109D) and imaged the cells 24-, 48- and 72-h post transfection in non-differentiating culture conditions. The T107/S109A–EGFP protein remained almost exclusively in the nucleolus throughout the duration of the experiment (Fig. 4b). The proportion of mutant protein localized in the nucleolus was significantly higher than in the nucleus, and this ratio of nucleolar–nuclear distribution differed significantly from controls (Fig. 4b). The relative proportion of TG cells in cultures expressing the T107/S109A mutant was also significantly reduced (Fig. 4c), although some TG cells were observed, which is probably attributable to endogenous wild-type Hand1 activity. By contrast, the T107/S109D mutant adopted a nucleus-wide localization and accordingly was able to promote TG cell differentiation (Fig. 4d). Aspartate substitutions serve as surrogates for phosphorylation, as has been demonstrated by mutation studies targeting helix 1 of the proto-oncogene Myc[31]. The T107/S109D mutant was not as efficient as wild-type Hand1 in promoting trophoblast differentiation (compare Fig. 4c with Fig. 3c), which is consistent with the reduced affinity of the aspartate mutant for E-factor heterodimerization and the correspondingly reduced biological activity as previously reported[30,32]. The precise nuclear localization of the T107/S109D mutant was distinct from wild-type Hand1–EGFP in that it localized to discrete, as-yet-unidentified nuclear foci (Fig. 2d).

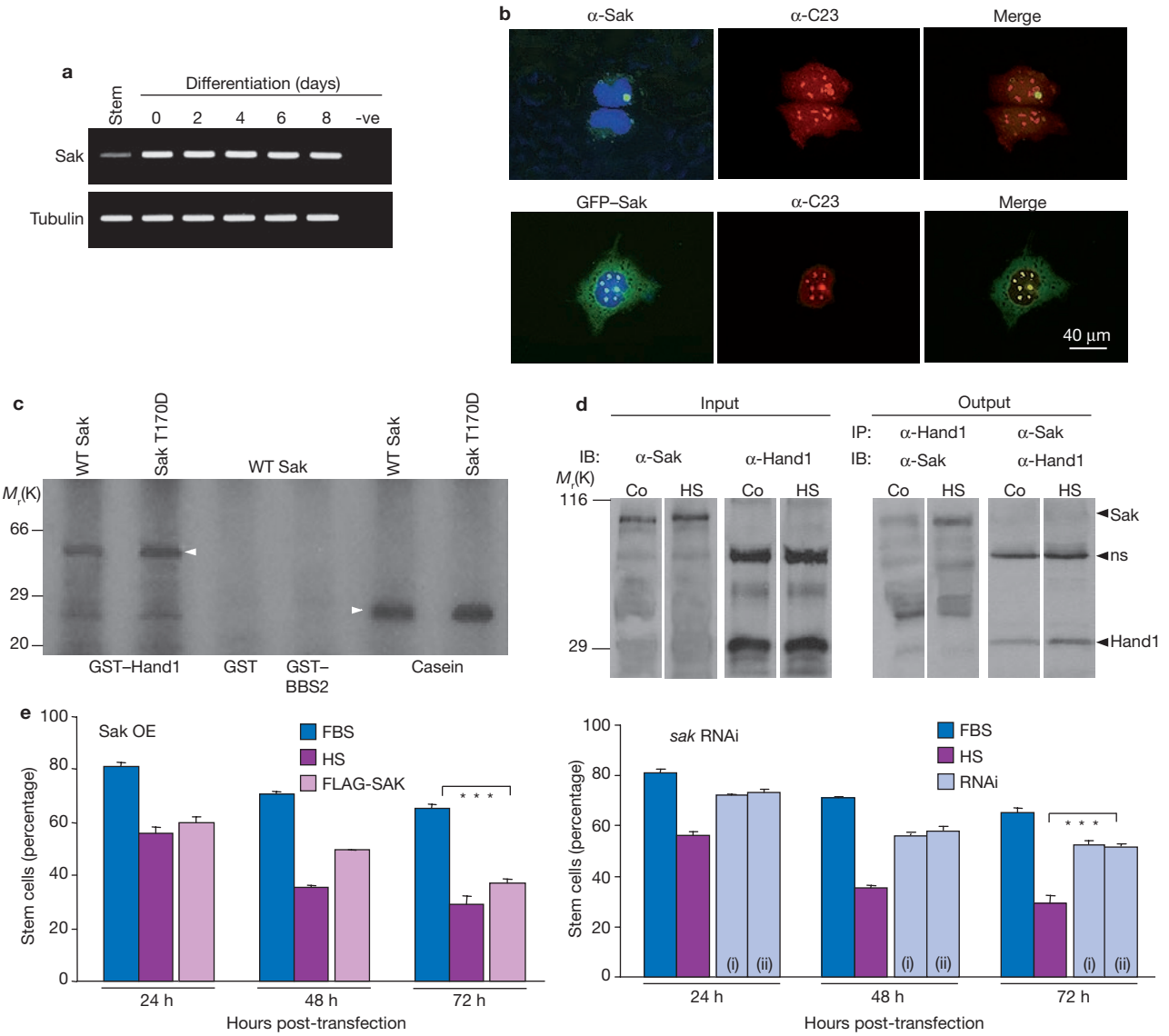

Figure 6 The polo-like kinase Sak (Plk4) localizes to Rcho-1 nucleoli at G2/M and phosphorylates Hand1. (**a**) Semi-quantitative RT–PCR reveals that Sak is upregulated at the onset of Rcho-1 differentiation induced by 10% HS (from the stem-cell stage to day 0) and maintained at high levels during differentiation. (**b**) Endogenous Sak localizes to a single nucleolus in Rcho-1 stem cells synchronized in G2/M following nocodozole treatment, whereas a GFP–Sak fusion protein is ectopically localized to multiple nucleoli with additional perinuclear fluoresence. (**c**) GST–Hand1 fusion protein is specifically phosphorylated *in vitro* by both wild-type Sak and an activating mutant T170D Sak (ref. 48) at levels comparable to the positive control substrate α-casein. (**d**) Coimmunoprecipitation of Hand1 with Sak in either Rcho-1 control (co; untreated and asynchronous cultures) or cells stimulated with 10% HS for 1 h reveals that Hand1 interacts with Sak *in vivo*. Note the stronger interaction in the HS-treated cells, which is consistent with a commitment towards a giant-cell fate. (**e**) Overexpression of Sak in Rcho-1 cells cultured in non-differentiating conditions (shown as FBS) significantly promotes TG-cell differentiation, whereas knockdown, using independent RNAi sequences of Sak, in stem cells cultured in horse serum significantly inhibits TG-cell differentiation. This is consistent with a requirement for Sak-mediated nucleolar release of Hand1 as a prerequiste for commitment of Rcho-1 stem cells towards a TG-cell fate; measurements in **e**; n=360 are mean±s.e.m, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; scale bars represent 40 μm. Full scans for **c** and **d** are presented in Fig. S4.

Confirmation that phosphorylation leads to nucleolar release of Hand1 was obtained following co-expression of the B56δ regulatory subunit of protein phosphatase 2A (PP2A) with Hand1–EGFP in Rcho-1 stem cells. Immunostaining demonstrated that B56δ is localized throughout the nucleus, including in the nucleolus, in Rcho-1 stem cells and that it exits the nucleus and becomes exclusively cytoplasmic during TG-cell differentiation (Fig. 5a). The transport of B56δ out of the nucleolus/nucleus during the onset of differentiation was confirmed following western-blot analysis on whole-cell lysates from stem and TG cells subtracted for the nucleolar fraction (Fig. 5b). B56δ was previously shown to dephosphorylate Hand1, and is downregulated during Rcho-1 differentiation[30]. We observed that B56δ overexpression in stem cells resulted in a failure of Hand1–EGFP to exit the nucleolus (Fig. 5c) and a significant reduction in TG-cell differentiation compared with Hand1–EGFP alone (compare Fig. 5c with Fig. 3c). In addition, overexpression of B56δ in Rcho-1 cells induced to differentiate inhibited TG cell formation (Fig. 5d) and was associated with a significant increase in nucleolar Hand1 (Fig. 5d; percentage of control cells with Hand

1 nucleolar localization is 43.9%±1.87 (mean±s.e.m. of three replicates after 72 h) compared with the percentage of overexpressed cells with nucleolar localization is 69.2%±2.56 (mean±s.e.m. of three replicates after 72 h); P=<0.001). Collectively, these studies suggest that phosphorylation of Hand1, through key residues in helix 1, is essential for its nucleolar release, subsequent heterodimer formation with nuclear E-factors (according to the classic bHLH paradigm of heterodimer formation with nuclear E-factors) and the activation of downstream target genes to promote stem-cell differentiation.

Sak (Plk4) phosphorylates Hand1 to initiate trophoblast differentiation

Previously PKC (protein kinase C) and PKA (protein kinase A) were implicated as kinases that could potentially phosphorylate Hand1 at T107 and S109 (ref. 30). Because these kinases do not localize to the nucleolus[33,34] and they are not present within the nucleolar proteome[34,35,36], it is unlikely that they are responsible for the phosphorylation and nucleolar release of Hand1 during Rcho-1 stem-cell differentiation. Moreover, because Rcho-1 stem cells exit from the mitotic cell cycle concomitantly with differentiation[37], we adopted a candidate-protein approach to identify an appropriate nucleolus-localized serine/threonine kinase that may simultaneously promote mitotic exit. A comprehensive search of the nucleolar proteome database (http://www.lamondlab.com/NOPdb/; ref. 35), along with associated expression studies in the trophoblast lineage, revealed a putative candidate in the non-canonical polo-like kinase, Plk4 (Sak; ref. 38). Sak has a functional polo-box (pb) domain that localizes the enzyme to the nucleolus during G2 (ref. 39) and is required for the anaphase-promoting complex (APC)-dependent destruction of cyclin B1 and exit from mitosis in the post-gastrulation embryo[39]. We determined that Sak is upregulated with the onset of differentiation (Fig. 6a). Moreover, expression of both endogenous Sak and ectopic GFP–Sak in G2/M synchronized cultures revealed nucleolar localization of Sak in Rcho-1 cells (Fig. 6b), which is consistent with the stage of the cell cycle during which both TS cells and Rcho-1 stem cells have been shown to exit the mitotic cell cycle and undergo endoreduplication[37]. In asynchronous Rcho-1 cells, GFP–Sak exhibited additional subcellular localization patterns (Supplementary Fig. S2a) equivalent to those reported previously in NIH-3T3 cells[39]. We subsequently tested the ability of Sak to phosphorylate Hand1 using an *in vitro* phosphorylation assay. Sak phosphorylated Hand1 to greater than 1 mol $Pimol^{-1}$ substrate suggesting it may act as a Hand1 kinase in a relevant physiological setting (Fig. 6c). Phosphorylation of Hand1 by Sak is consistent with the presence of degenerate phosphorylation consensus sites for Sak located at T107 and S109 in helix 1 of Hand1 (ref. 40). Moreover, overexpression of Sak in Rcho-1 cells resulted in enhanced phosphorylation of endogenous Hand1 *in vivo* (Supplementary Fig. S2b). Notably, Sak failed to enhance phosphorylation of either the T107/S109A or T107/S109D mutant EGFP fusion proteins in the same assay, suggesting that the kinase is acting in a site-specific manner at the predicted T107 and S109 residues in helix 1 (data not shown). Consistent with these findings, we were able to immunoprecipitate endogenous Hand1 with Sak, and vice versa, in Rcho-1 cells where, appropriately, the interaction was enhanced when cells were induced to differentiate (Fig. 6d; compare lane 5 with 6 and lane 7 with 8).

We next assessed the effects of a gain and loss of Sak function on Rcho-1 differentiation. Overexpression of Sak (Flag–Sak; expression determined by α-Flag, rhodamine-conjugated positive cells) in non-differentiating conditions significantly increased Rcho-1 differentiation to a level equivalent to that induced by horse serum (Fig. 6e), whereas during differentiating conditions, RNAi knockdown of Sak, using two independent RNAi sequences (see Supplementary Fig. S2d for levels of Sak knockdown) significantly blocked Rcho-1 differentiation (Fig. 6e). Sak overexpression was associated with reduced numbers of stem cells and a significant increase in nuclear Hand1 (Fig. 6e; percentage of control cells with nucleus-wide Hand 1 localization is 28.6±2.08 (mean±s.e.m. of three replications at 72 h) versus percentage of overexpressed cells with nuclear-wide Hand1 localization is 54.1±2.78 (mean±s.e.m. of three replications at 72 h); P=<0.001) whereas knockdown of Sak resulted in reduced numbers of giant cells and increased nucleolar Hand1 (Fig. 6e; percentage of cells with nucleolar Hand1 localization: control cells, 43.9±1.87 (mean±s.e.m.); RNA(i), 68.9±2.39 (mean±s.e.m.); RNAi (ii), 69.8±1.44 (mean±s.e.m.); mean of three replications after 72 h were used; P=<0.001). Sak knockdown also resulted in significantly reduced Hand1 phosphorylation in differentiating conditions (Supplementary Fig. S2b).

Sak is essential for Hand1-induced secondary giant-cell differentiation

Finally, we investigated the phenotype of Sak-null embryos in terms of trophoblast differentiation and potential mislocalization of Hand1 *in vivo* (Fig. 7). Initial observations at E7.5 revealed reduced overall size of concepti (Fig. 7a, b) and a markedly enlarged ectoplacental cone (EPC) in Sak-null embryos compared with heterozygous controls (Fig. 7c, d), consistent with an expansion in the diploid source of trophoblast 'stem cells' and reduced TG-cell differentiation. DNA staining of histological sections (Fig. 7e–j) revealed that the differentiation of primary giant cells (PGC), which arise directly from trophectoderm at the blastula stage, was normal in Sak-null mutants (Fig. 7g, h), whereas the secondary giant cell (SGC) population, which arises from the outer layer of the EPC, was significantly reduced with associated failed migration through the overlying maternal decidua (Fig. 7i, j). PGC and SGC populations were distinguished by immunostaining for prolactin-like protein A (PLP-A; see Fig. S3a), which is expressed exclusively in secondary TG cells[41]. Comparable plane sections through E7.5 embryos revealed 63±9.6 (mean±s.e.m.) SGCs in controls versus 21±5.2 (mean±s.e.m.) SGCs in Sak-null embryos. Six replica sections for each genotype were used. The observation of reduced SGC differentiation in Sak-null embryos is consistent with the phenotype previously described for the Hand1 mutants[5] in which the embryos survive to around E7.5, begin to gastrulate and establish an implantation chamber through PGC migration but have significantly reduced numbers of SGCs[5]. However, Sak mutants do not exactly phenocopy Hand1 mutants, consistent with the fact that not all of Hand1 function during early embryogenesis is mediated through Sak, and vice versa. Indeed Hand1 is required for proliferation and maintenance of the EPC, by an as-yet-unknown mechanism[8], whereas Sak is required for mitotic cell-cycle exit. Consequently loss of Sak results in expanded populations of mitotically active cells, including those of the EPC[39]. Thus the crucial finding in the context of this study is that in both genetic backgrounds, commitment of trophoblast precursors to a SGC fate is impaired. Investigating the subcellular localization of Hand1 in a Sak-null background (Fig. 7k–t) revealed that Hand1 was nuclear in the majority of SGCs in the control embryos (Fig. 7k–n), whereas in

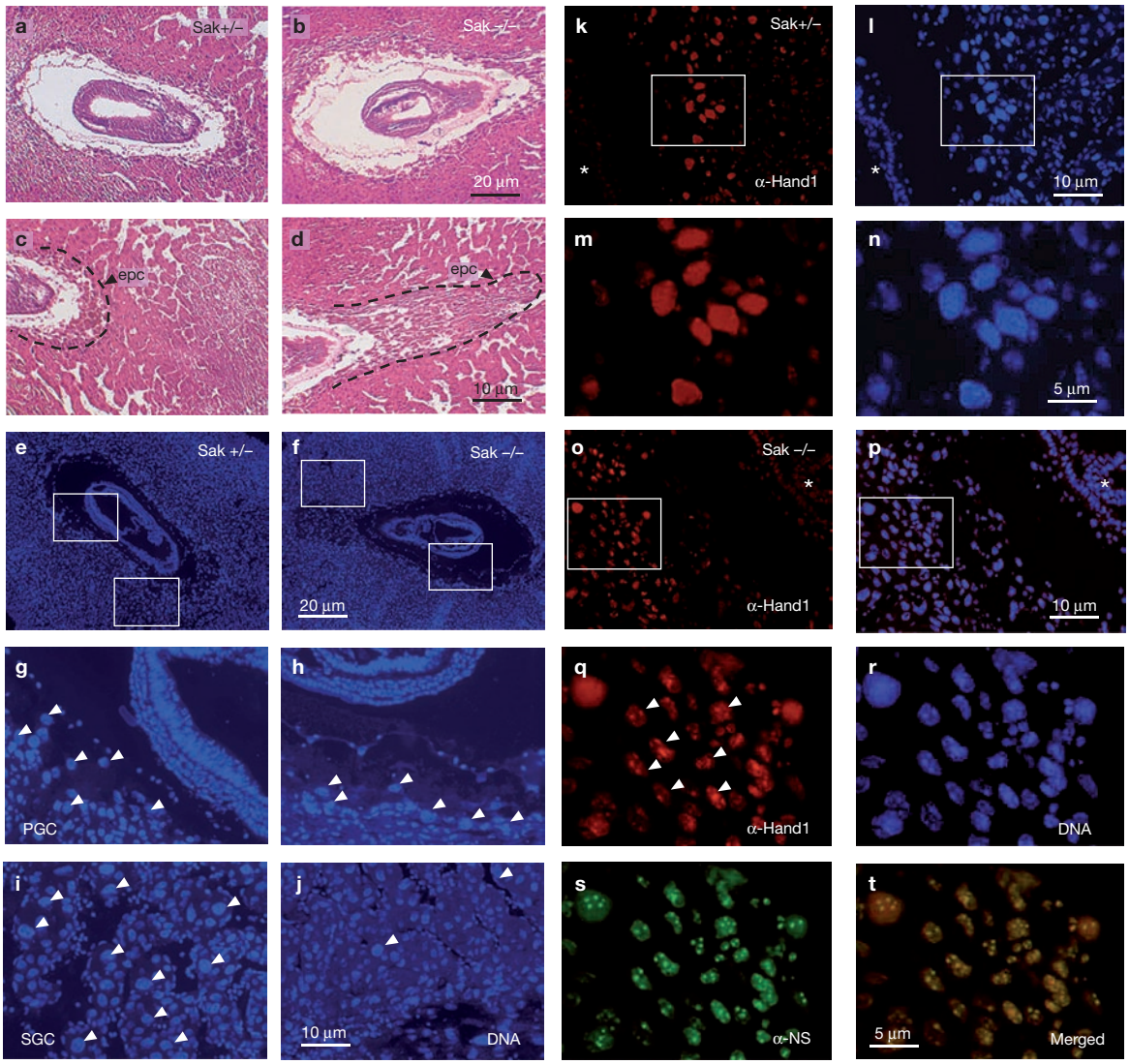

Figure 7 Sak-null embryos reveal an expanded diploid trophoblast population that significantly reduced secondary giant-cell differentiation and nucleolus-restricted Hand1 localization. Histological sections (**a**–**d**) through Sak heterozygous (**a**, **c**: +/–) and homozygous mutants (**b**, **d**; –/–) at E7.5 reveal that Sak-null embryos are smaller than their heterozygous littermates (**b**) and have a significantly expanded ectoplacental cone (epc) of diploid trophoblast cells (**d**). DNA staining of sections (**e**–**j**; the boxes in **e** are magnified in **g** and **i**, and the boxes in **f** are magnified in **h** and **j**) reveals that Sak mutants have equivalent levels of primary trophoblast giant cells (PGCs; **g**, **h**) but significantly reduced the number of secondary giant cells (SGCs; **i**, **j**) compared with heterozygous littermates. White arrowheads identify PGCs in **g** and **h** and SGCs in **i** and **j**. Immunostaining for Hand1 (**k**–**r**; the box in **k** is magnified in **o** and the box in **l** is magnified in **p**) reveals nucleus-wide localization of Hand1 in secondary giant cells in heterozygous control embryos (**k**, **m**) but nucleolar restriction of Hand1 in the expanded diploid trophoblast population in the Sak-null embryos (**o**, **q**) as confirmed by counterstaining for the nucleolar-specific marker nucleostemin (NS; **s, t**). Note the requirement for Sak to induce nucleolar release of Hand1 and appropriate SGC differentiation *in vivo*. White asterisks in **k**, **l**, **o**, **p** reveal the relative position of the most rostral part of the embryo in section. White arrowheads in **q** highlight mutant cells with exclusively nucleolar Hand1. Scale bars represent 5 μm in **m**, **n**, **q**, **r**, 10 μm in **c**, **d**, **g**–**j**, **k**, **l**, **o** and **p** and 20 μm in **a**, **b**, **e** and **f**.

the expanded diploid trophoblast of Sak-mutants, Hand1 remained predominantly nucleolar, which is coincident with rare commitment to a SGC fate (Fig. 7q–t). The mislocalization of Hand1 in SGC precursors as well as the differential effect on SGC versus PGC differentiation in Sak-deficient embryos is also consistent with the *in vitro* studies, because Rcho-1 differentiation is thought to most closely resemble SGC differentiation[25], an observation further supported by the expression of PLP-A in Rcho-1 giant cells (see Supplementary Fig. S3b). Collectively, the *in vitro* and *in vivo* studies confirm a role for Sak-induced phosphorylation in driving the nucleolar–nuclear release of Hand1 as a prerequisite for TG-cell differentiation and placentation.

DISCUSSION

Our results describe a novel cellular mechanism for regulating the activity of a transcription factor as a key determinant of a stem-cell lineage. We have identified a negative regulator of Hand1, an I-mfa-domain-containing protein, that anchors Hand1 in the nucleolus. Sequestration of Hand1 in the nucleolus, during trophoblast stem-cell renewal, is superseded by Sak-induced phosphorylation of Hand1 to bring about its release into the nucleus and commitment to differentiate towards a TG-cell fate. Thus, we present the first example of phosphorylation leading to the nucleolar release of a critical factor in a mammalian cell lineage. Moreover, we demonstrate that recruitment of a polo-like kinase, along with a

specific regulatory subunit of the phosphatase PP2A[42], to regulate nucleolar release is conserved throughout evolution, suggesting that phosphorylation acts as a widespread mechanism for the release of proteins from nucleolar storage.

In this regard, the nucleolus can no longer be thought of simply as a ribosome biosynthesis factory[43]. The previously published nucleolar proteome revealed a flux of endogenous proteins through the nucleolus[35], with the partition of proteins between the nucleolus and nucleoplasm reflecting the general physiological state of the cell. Our study uniquely reveals the functional implication of partitioning, whereby the nucleolus acts as a molecular 'sink' to retain Hand1 as a key factor implicated in the commitment to differentiation within a cell lineage. Given that the nucleolar proteome contains a dynamic profile of at least 489 nucleolar proteins, of which a significant proportion are known to be associated with transcription[35], compartmentalization within the nucleolus is likely to represent a widespread mechanism for regulating gene expression and cellular events in a variety of lineages. □

METHODS

Constructs and cell lines. For the yeast two-hybrid bait, full-length Hand1 cDNA was cloned into pGBDU kindly provided by Stanley Hollenberg (Fred Hutchinson Cancer Research Center, Seattle, WA). GST–Hand1, pcDNA3–Hand1, pFlag–Hand1, pcDNA3–E12 and the mock Hand1 target-gene reporter have been previously described[44]. pcDNA3/HIS-HICp40, pcDNA3/His HIC-p32, pcDNA3–His–HICΔ, EGFP–HICp40 and EGFP–HICp32 were kind gifts from Sabine Thebault (Institut de Biologie, Montpellier, France). Hand1 was cloned by PCR from a construct previously described[44] and inserted into pEGFP-N1 (Clontech, Palo Alto, CA). Hand1 point mutations used — Hand1 S109A/T107A and S109D/T107D — were kind gifts from Anthony Firulli (Herman Wells Centre, James Whitcomb Riley Hospital for Sick Children, Indianapolis, IN) and were cloned into pEGFP-N1 (Clontech). Hand2–EGFP was cloned using PCR from pHAND2-neo and inserted into pEGFP-N1 (Clontech). MyoD–EGFP was described previously[45] and kindly provided by Vivek Mittal (Cancer Genome Research Centre, Cold Spring Harbor Laboratory, Woodbury, New York). B56δ in the expression vector pCEP4.1 was described previously[30] and was also provided by Anthony Firulli. pFlag–Sak, Flag–SakT170D (activating) and Flag–SakK41M (inactivating) were kindly provided by James Dennis (Samuel Lunenfeld Research Institute, Toronto, Canada), and pEGFP–SAK was kindly provided by Frank Sicheri (Samuel Lunenfeld Research Institute, Toronto, Toronto, Canada). For the RNAi constructs, 21-bp or 22-bp sense and antisense *HICp40* and *Sak* sequences were inserted downstream of the H1 RNA pol III promoter in a modified pcDNA3 vector as described previously[46]. Sequences of the shRNAs for HICp40shRNA1 and HICp40shRNA2 were AATCCTTCAGCTGGTGAACTT and CAGTCCAGCTTGTCTGTAAACA for nucleotides 190–210 and 409–430, respectively (accession number NM_175088, *Mus musculus* MyoD family inhibitor domain containing (Mdfic), GI: 88014734; 100% homology to corresponding rat ESTs). SakshRNA1 and SakshRNA2 were AACAGAGATTTCCAGGACTAT and AATACTGGCGGAAATATCAGT, corresponding to nucleotides 1590–1610 and 2122–2142, respectively (accession number XM_227064, *Rattus novegicus* polo-like kinase 4, GI: 10946713).

NIH3T3 cells were cultured as described previously[44], were co-transfected with Effectene™ as per the manufacturer's instructions (Qiagen, Valencia, CA) and luciferase reporter activity measured as described previously[44]. Rcho-1 cells, a kind gift from Michael Soares (Division of Cancer and Developmental Biology, University of Kansas, Lawrence, KS) were cultured and induced to differentiate according to the method described previously[4,26]. TS cells were a kind gift from Satoshi Tanaka (National Institute for Basic Biology, Okazaki, Aichi, Japan) and were cultured as previously described[28]. Rcho-1 and TS cells were transfected with Lipofectamine as per the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Yeast two-hybrid. Standard yeast genetic techniques and growth media were used, and the two-hybrid screen was carried out as described previously using a mouse E9.5/10.5 library[10].

GST pulldown assay. GST and GST fusion proteins were expressed and purified according to the manufacturer's protocol (Amersham Pharmacia, San Francisco, CA). GST pulldown assays were carried out as described previously[44] using GST–Hand1 and HICp40, HICp32 and HICΔC plasmids processed for *in vitro* transcription and translation using a TNT kit (Promega, Madison, WN) in the presence of [^{35}S] methionine.

Antibodies and immunofluorescence. Cells were fixed and immunostained as described previously[44], and fluorescent images either for EGFP or fluorescein/rhodamine-conjugated-antibody staining were obtained using a Zeiss Axio Imager M1 microscope (Zeiss, Hamburg, Germany).

Primary antibodies used were polyclonal α-B56δ (Stratagene, La Jolla, CA), α-Hand1 (Santa Cruz Biotechnology, Santa Cruz, CA; Abcam, Cambridge, UK), α-HIC (kind gift of Jean-Michel Mesnard, Laboratoire Infectiones, Retrovirales et Signalisation Cellulaire, Institut de Biologie, Montpellier, France), α-Plk4 (Abcam), α-PL-1 (Chemicon, Temecula, CA), α-PLP-A (Chemicon), α-Nucleolin (C23; Santa Cruz) and α-Nucleostemin (NS; R&D Systems, Minneapolis, MN) were used at dilutions between 1:100 and 1:300. Rhodamine-phalloidin (Molecular Probes, Carlsbad, CA) was used at a final concentration of 5 units (165 nM).

Reverse-transcribed PCR, northern and western analyses. Rcho-1 cells were grown in either 20% FBS or 10% horse serum supplemented with] NCTC 135 (Invitrogen) for the indicated time. RNA was extracted using a Micro FastTrack 2.0 kit (Invitrogen). RNA was DNase treated before reverse transcription was performed using Superscript II RT (Invitrogen) according to the manufacturer's instructions. PCR was performed using standard conditions with primers specific for Sak/PLK4 (sense: 5′-GCAGGAGTATCTTCCATCAG-3′; antisense: 5′-GGAGTTGGATTAGAAAACAT-3′) and tubulin (sense: 5′-TCACTGTG CELLSCTGAACTTACC-3′; antisense: 5′-GGAACATAGCCGTAAACTGC-3′). Northern analysis was carried out using a NorthernMax™ kit (Ambion, Foster City, CA) according to the manufacturer's instructions and probed with a cDNA for Hand1. Protein was extracted using standard protocols[44], and nucleolar fractions were isolated using NP-40 as described previously[47]. Western blots were probed with α-Hand1 (1:200; Santa Cruz), α-HIC (1:500)[11], α-Plk4 (1:200, Abcam), α-B56δ (1: 5000, Stratagene), α-phosphoserine (1:150, Abcam), α-Flag M2 (1:700, Sigma, St Louis, MO), α-HIS (1:1000, Abcam) and α-Gapdh (1:1000, Chemicon, Temecula, CA).

***In vitro* phosphorylation.** Rcho-1 cells were transfected with either pFlag–Sak or pFlag–SakT170D (activating mutant)[48] and lysates immunoprecipitated using a α-Flag M2 antibody (Sigma) as described previously[44]. Precipitates were incubated with either 30 μg of GST–Hand1 or lysates from Hand1–EGFP or T107/S109A/D–EGFP-transfected Rcho-1 cells and 2 μCi of 30 Ci mM^{-1} ^{32}P γdATP for 30 min in kinase buffer (50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT). α-Casein was used as a positive control as described previously[48]. Labelled protein was run through a 12% SDS polyacrylamide gel, washed in TBS and dried and exposed to autoradiography film.

Immunoprecipitation. Rcho-1 cells were treated for 1 h with 10% horse serum to induce a commitment towards differentiation, lysed in RIPA buffer and immunoprecipitated with pre-blocked protein sepharose-A/G beads conjugated with 3–4 μg of polyclonal α-PLK4 antibody for 2 h. Controls were untreated asynchronous Rcho-1 cell lysates. Samples were washed in PBS, boiled in loading dye and run through an SDS–PAGE followed by western analysis for Hand1 as above.

Sak-deficient embryos. Sak-null embryos at E7.5 were fixed, either dehydrated and wax-embedded or cryo-embedded as previously described[5,49] and serially sectioned to 10 μm thickness. Genotyping by nested PCR using primers previously described[39] was carried out on tissue taken from wax-embedded sections[5]. Sections were either dewaxed and rehydrated or thawed in PBS and stained with haematoxylin and eosin for histology or with antibodies against α-Hand1 (Abcam), α-Nucleostemin (NS; R&D Systems) and α-Plk4 (Abcam; to confirm the PCR genotyping of Sak-null embryos) for immunofluoresence and counterstained with the nuclear marker bis-benzamide as previously described[44,50].

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

D.M.J.M carried out the functional cell-based characterization studies. C.A.R. and N.S. contributed to northern and western data and histological sections from Sak-null embryos. M.D.M.F.-V. peformed the yeast two-hybrid screen. COR collected and prepared Sak-null embryos. C.J.S. and J.W.D. provided Sak-null embryos and critical appraisal of Sak-null embryo data. P.R.R. carried out initial characterization studies and Sak-null embryo analyses, devised the functional studies and wrote the manuscript.

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