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Functional regulation of oestrogen receptor pathway by the dynein light chain 1

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Overexpression and phosphorylation of dynein light chain 1 (DLC1) have been shown to promote the growth of breast cancer cells. However, the role of DLC1 in the action of the oestrogen receptor (ER) remains unknown. Here, we found that oestrogen induces the transcription and expression of DLC1. DLC1 facilitated oestrogen-induced ER transactivation and anchorage-independent growth of breast cancer cells. We show that DLC1 interacts with ER, and such interaction is required for the transactivation-promoting activity of DLC1. Further, DLC1 expression led to enhanced recruitment of the DLC1–ER complex to the ER-target gene chromatin. Conversely, DLC1 down-regulation compromised the ER-transactivation activity and also its nuclear accumulation, suggesting a potential chaperone-like activity of DLC1 in the nuclear translocation of ER. Together, these data define an unexpected upregulation of DLC1 by oestrogen and a previously unrecognized DLC1–ER interaction in supporting and amplifying ER-initiated cellular responses in breast cancer cells.

Keywords: breast cancer cells; dynein; oestrogen receptor; gene expression; signalling

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INTRODUCTION

Cellular processes that depend on cytoskeleton remodelling, such as vesicle and membrane transport, are influenced by dynein, a multiprotein molecular motor complex that has been shown to regulate cytoplasmic organelle transport, the movement of chromosomes, the assembly and positioning of mitotic spindles and nuclear migration (Vaisberg *et al*, 1993; Holzbaur & Vallee, 1994). Dynein light chain 1 (DLC1), an 8-kDa component of the cytoplasmic dynein complex, is a minus-end-directed microtubule-based motor that transports cargo along microtubules (Hirokawa, 1998; Pazour *et al*, 1998). DLC1 also exerts a chaperone-like function in combination with the intermediate chain IC74 of the dynein motor complex (Makokha *et al*, 2002). DLC1 has an essential role in living cells, as mutations in its *Drosophila* homologue (*ddlc1*) cause morphogenetic defects and apoptotic cell death in *Drosophila melanogaster* (Dick *et al*, 1996). DLC1 is highly conserved among species, participating in nuclear interaction with transcription factors such as the erect wing gene product (EWG) of *Drosophila* and the structurally and functionally related nuclear respiratory factor 1 (NRF1) in humans (Herzig *et al*, 2000). Recently, DLC1 phosphorylation by p21-activated kinase 1 (Pak1; Vadlamudi *et al*, 2004), another cytoskeleton-remodelling kinase downstream of growth factors and oestrogen signalling (Bokoch, 2003; Mazumdar & Kumar, 2003), has been shown to promote the survival and growth of oestrogen receptor (ER)-positive breast cancer cells.

The biological effects of oestrogen result from its binding to the structurally and functionally distinct ERs (ERα and ERβ). ERα (denoted ER here) is the principal ER in the human mammary epithelium and regulates gene expression in both a ligand-dependent and a ligand-independent manner. To exert its transactivation functions, the activated ER translocates to the nucleus and binds a palindromic sequence (Herynk & Fuqua, 2004). In addition to oestrogen binding, the transactivation function of ER is modified by coactivators and chromatin-remodelling complexes (Kumar *et al*, 2004). The molecular mechanisms underlying the transport of ER to the nucleus are not completely understood at present. Because most DLC1 is not bound to microtubules and because DLC1 interacts with unrelated cellular proteins (Benashski *et al*, 1997), DLC1 has been proposed to influence cellular physiology both dependent and independent of the motor function. Despite the widely acknowledged role of DLC1 in retrograde transport and in nuclear migration (Beckwith *et al*, 1998; Pazour *et al*, 1998), its role in the action of ER remains unknown. Here, we explored the role of DLC1, a component of the dynein motor complex, in the action of ER in breast cancer cells and the nuclear transport of ER in these cells. Our results define an unexpected, chaperone-like function of DLC1 in the action of ER and a functional DLC1–ER interaction

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in supporting and amplifying ER-initiated cellular responses in mammalian cells.

RESULTS AND DISCUSSION

Oestrogen induces transcription and expression of DLC1

Recent findings have suggested that DLC1 is overexpressed in breast cancer and that DLC1 phosphorylation by Pak1 promotes the survival and growth of ER-positive breast cancer cells (Vadlamudi *et al*, 2004). Because Pak1 is also activated by oestrogen (Mazumdar & Kumar, 2003), we initially explored the possibility that DLC1 is expressed through an ER pathway. We found that oestrogen induces the expression of DLC1 messenger RNA in a dose- and time-dependent manner and that this induction could be effectively blocked with the pure anti-oestrogen ICI 182,780 as well as with actinomycin D, an inhibitor of transcription (Fig 1A,B). To determine whether the observed upregulation of DLC1 by oestrogen was transcriptional in nature, we analysed the sequence of the putative DLC1 promoter for the presence of oestrogen-response elements (EREs; GenBank genomic sequence accession number NM 003746 using MatInspector software GmbH; Genomatix, München, Germany). This program did not show any consensus 13-base-pair (bp) ERE sites. However, we did notice that the DLC1 promoter contained potential ERE half-sites (TGACC), each in the vicinity of AP1-binding sites (supplementary Fig S1 online). To learn more about the function of these sites, we next cloned the DLC1 promoter 2.4-kb fragment containing the ERE half-sites into a pGL3-luciferase (luc) reporter system and found that oestrogen indeed stimulates DLC1 promoter activity in MCF-7 and Ishikawa cells (Fig 1C). The observed oestrogen-mediated increase of DLC1 mRNA was also accompanied by an increase in DLC1 protein in ER-positive MCF-7 and Ishikawa cells (Fig 1D).

Signalling requirement of oestrogen induction of DLC1

Next, we explored the potential role of Pak1 signalling in the upregulation of DLC1 by oestrogen. We used a previously characterized MCF-7 clone (MCF-7/DA-Pak1 cells) that expresses catalytically active Pak1 under an inducible Tet-on promoter and found that conditional expression of Pak1 upregulated the expression of DLC1 mRNA as well as that of protein (Fig 2A). Conversely, a previously characterized MCF-7 clone expressing the dominant-negative autoinhibitory Pak1 fragment amino acid 83–149 (MCF-7/DN-Pak1 cells) downregulated DLC1 expression at the mRNA as well as protein levels (Fig 2B). Consistent with these results, conditional knockdown of Pak1 by Pak1-siRNA (short interference RNA) also resulted in the loss of DLC1 expression, in both the absence and the presence of oestrogen (Fig 2C). These results suggest that the optimal expression of DLC1 depends on the presence of functional Pak1, and that DLC1 is a common target of oestrogen. As expected, we also found that wild-type Pak1 stimulated DLC1 promoter activity, whereas expression of the Pak1 inhibitor 83–149 suppressed DLC1 promoter activity (Fig 2D). These findings show that Pak1 signalling has an important role in the regulation of DLC1 expression by oestrogen.

DLC1 overexpression promotes oestrogen responsiveness

To understand the significance of oestrogen regulation of DLC1, we explored whether DLC1 overexpression is sufficient to modulate hormone response. For this purpose, we generated MCF-7 cells expressing T7-DLC1 under a tetracycline-inducible promoter (MCF-7/DLC1 cells; Fig 3A). Surprisingly, by inducing the expression of DLC1, these MCF-7/DLC1 cells showed an increased expression of the ER-regulated cell-survival protein Bcl-2 (Fig 3B), implying that DLC1 overexpression has a role in the enhanced responsiveness to oestrogen. To implicate further the endogenous DLC1 in the induction of ER-regulated genes, we examined the effect of inhibition of the endogenous DLC1 by siRNA on ER-regulated genes. We found a significant reduction in the ability of oestrogen to induce the expression of progesterone receptor (PR) and cathepsin D in cells treated with DLC1 siRNA as compared with cells treated with control siRNA (Fig 3C). To implicate DLC1 directly in the responsiveness to oestrogen, we examined how the conditional overexpression of DLC1 affected the ability of oestrogen to influence ER transactivation and cell growth. Results showed that DLC1 overexpression caused a significant increase in the ability of oestrogen to stimulate

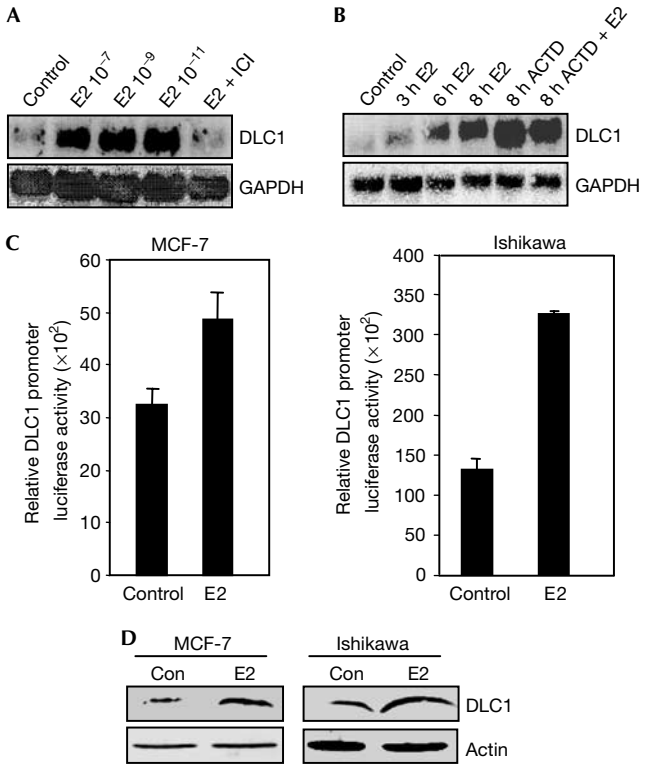
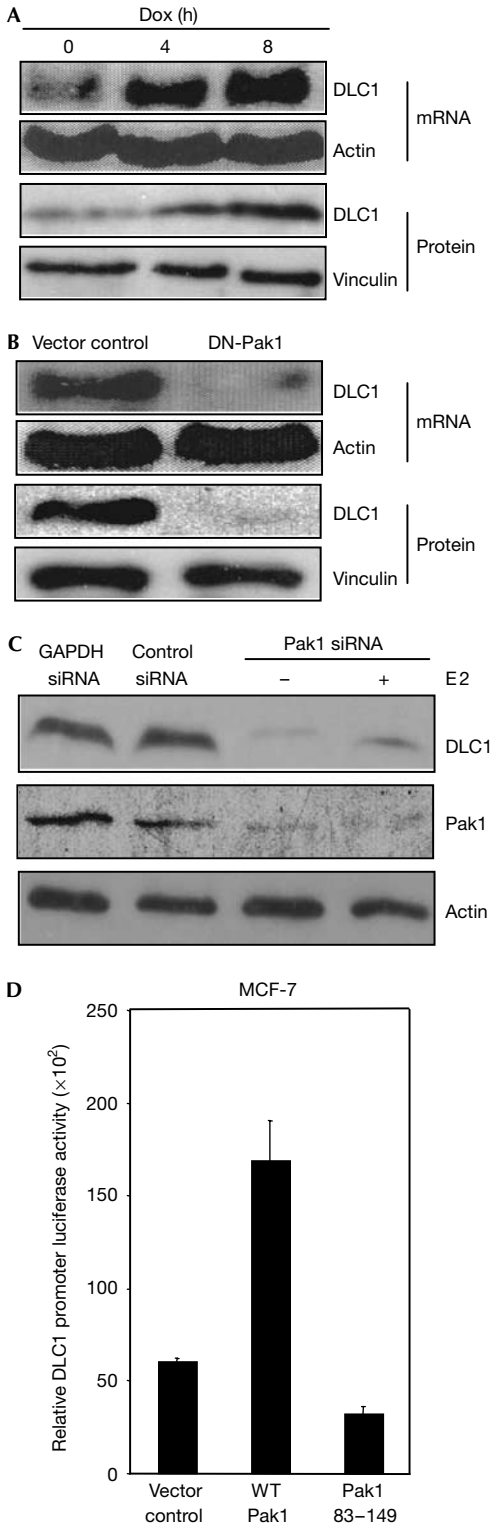


Fig 1 | DLC1 is an E2-responsive gene. **(A)** DLC1 mRNA is increased after oestrogen stimulation. MCF-7 cells were treated with different concentrations of E2 for 16 h and analysed by northern blotting. **(B)** Effect of actinomycin D on the ability of E2 to upregulate DLC1 mRNA in MCF-7 cells. Cells were collected at different time points after treatment with E2 and/or actinomycin D (10 μg/ml) and DLC1 mRNA was analysed by northern blotting. **(C)** Upregulation of DLC1 promoter activity in MCF-7 and Ishikawa cells treated with E2 for 24 h. Cells were transfected with ERE-luc for 24 h, treated with E2 for 24 h and ERE-luc activity was measured. **(D)** Upregulation of DLC1 protein in MCF-7 and Ishikawa cells treated with E2 for 24 h.

ERE-dependent transactivation in MCF-7 cells and Ishikawa cells (Fig 3D). In addition, DLC1 expression also potentiated the ability of oestrogen to stimulate cell growth and promoted anchorage-independent growth (Fig 3E,F). To validate further a role for DLC1 in the regulation of ER transactivation and cell growth, we downregulated endogenous DLC1 by using siRNA, and found a significant reduction in the growth rate of MCF-7 cells (Fig 3G) as well as ER transactivation (Fig 3H). These results suggest that the observed DLC1–ER interaction could constitute one of the mechanisms by which the transactivation activity of ER is enhanced by DLC1 and/or its putative binding partners.



DLC1, a unique ER-interacting protein

As DLC1 participated in the transactivation functions of ER (Fig 3), we suspected that DLC1 could interact with ER and thus modulate the ER-transactivation function. To test this, we first evaluated the ability of *in vitro*-translated ER protein to bind to DLC1–GST (glutathione-*S*-transferase) fusion protein. Indeed, the DLC1–GST fusion protein, but not GST, efficiently interacted with ^{35}S-labelled, full-length ER protein (Fig 4A). The DLC1-binding site in ER was mapped to the AB domain (amino acids 1–180) of ER (Fig 4B). Using a series of DLC1 deletion constructs, we identified the minimal region of DLC1 required for its interaction with ER (amino acids 67–89; Fig 4C). The *in vivo* interaction of endogenous DLC1 with ER was confirmed by co-immunoprecipitation of ER and DLC1 from lysates of exponentially growing cells by using an anti-ER monoclonal antibody but not by control IgG (Fig 4D). To validate these findings and determine the effect of oestrogen on the DLC1–ER interaction, we next immunoprecipitated ER from cell lysates from MCF-7/DLC1 cells treated or not treated with doxycycline (Dox) to induce the expression of T7-DLC1 and then stimulated with oestrogen. Results showed that DLC1 upregulation was sufficient to promote DLC1–ER interaction and that this interaction was further increased by oestrogen (Fig 4E). To investigate whether the direct binding of DLC1 to ER is important for the transactivation capabilities of ER, we studied the effect of the carboxy-terminal deletion DLC1 mutant on ER transactivation activity. We found that DLC1, but not DLC1 mutant, was able to enhance ER transactivation (Fig 4F), suggesting that DLC1–ER interaction might be important in the noted ER-transactivation function of DLC1.

DLC1 interacts with ER and the ER-target gene chromatin

To delineate the physiological significance of the DLC1–ER interaction, we investigated whether DLC1, alone or as a part of the DLC1–ER complex, is also recruited to the ER-target *pS2* gene promoter chromatin, using a chromatin immunoprecipitation (ChIP) assay. As expected, oestrogen stimulation of the MCF-7/DLC1 (Dox-untreated) cells resulted in significant recruitment of

◀ **Fig 2** | Signalling pathways in oestrogen regulation of DLC1. (**A**) Increased expression of DLC1 mRNA and protein in MCF-7/DA-Pak1 cells. Cells were induced with Dox, and DLC1 mRNA and protein levels were assessed. (**B**) Decreased expression of DLC1 mRNA and protein in MCF-7/DN-Pak1 cells. Cells were induced for 8 h with Dox and DLC1 mRNA and protein levels were assessed. (**C**) Downregulation of DLC1 mRNA in MCF-7 cells treated with siRNA-Pak1 for 24 h. (**D**) Modulation of DLC1 promoter activity in MCF-7 cells by the expression of Pak1 or DN-Pak1 for 24 h. WT, wild type.

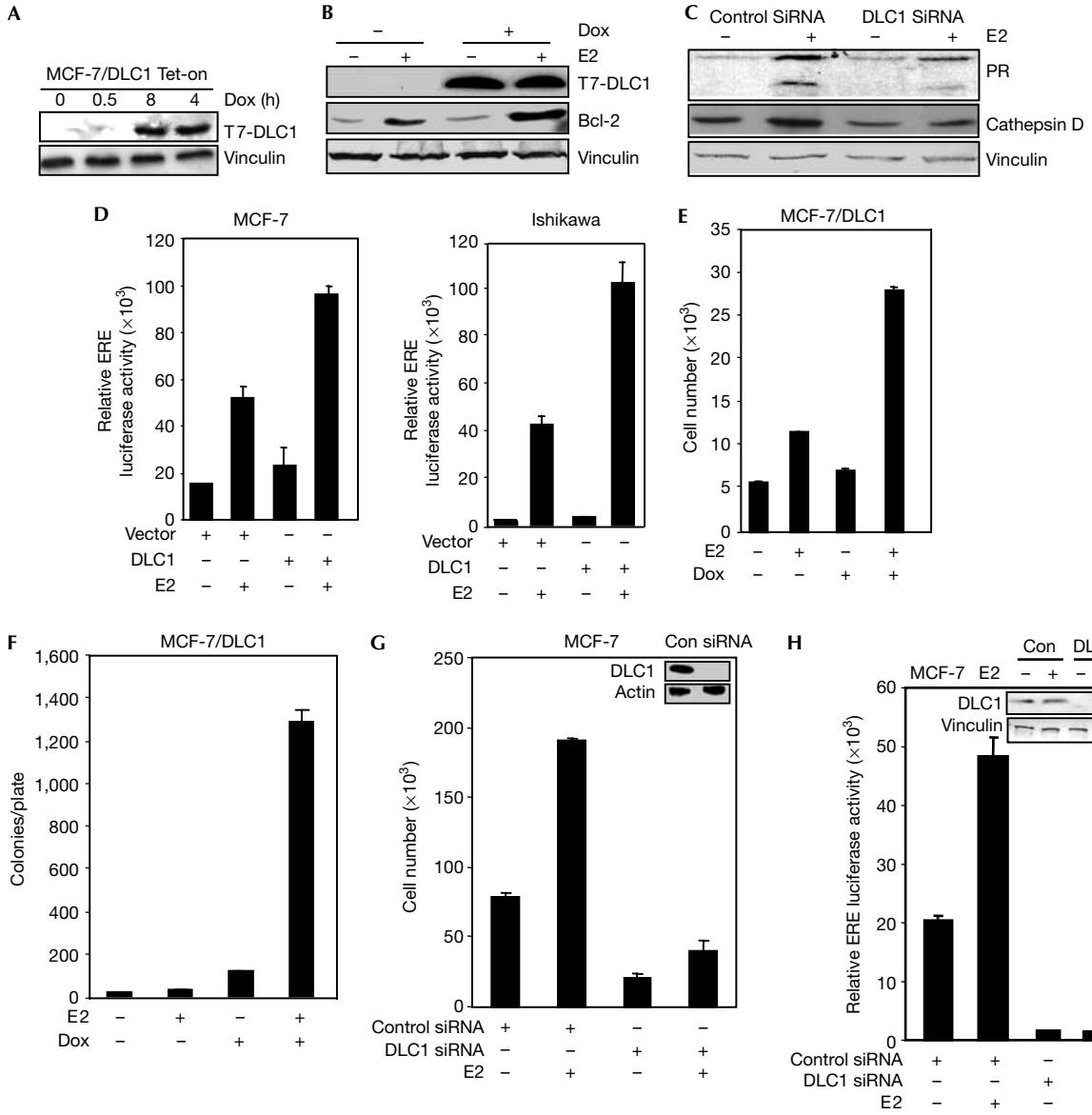
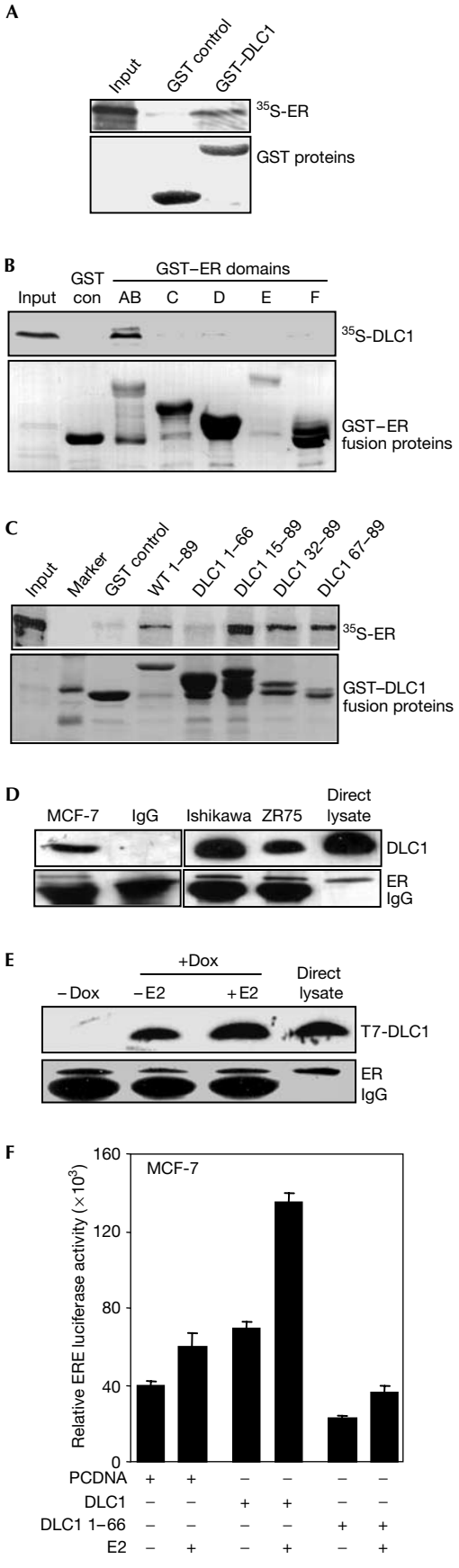


Fig 3 | DLC1 overexpression leads to E2 hypersensitivity. (**A**) Dox-mediated upregulation of T7-DLC1 expression in MCF-7/DLC1 clone. (**B**) DLC1 overexpression potentiates Bcl-2 expression by E2. MCF-7/DLC1 cells were treated with or without Dox and stimulated with E2 for 24 h. (**C**) DLC1 knockdown reduces the expression of progesterone receptor (PR) and cathepsin D. Cells were treated with control or DLC1-specific siRNA for 48 h and stimulated with E2 for 24 h. Then, lysates were immunoblotted with the indicated antibodies. (**D**) DLC1 overexpression potentiated E2-mediated stimulation of ERE-luc activity in MCF-7 and Ishikawa cells. MCF-7 and Ishikawa cells were transfected with ERE-luc, treated with E2 for 24 h and ERE-luc activity was measured. (**E**) DLC1 upregulation potentiates growth stimulation by E2. MCF-7/DLC1 cells were treated with or without Dox and stimulated with E2 for 5 days. (**F**) DLC1 upregulation promoted anchorage-independent colony formation. MCF-7/DLC1 cells were plated in soft agar, treated or not treated with Dox and stimulated with E2 for 21 days. (**G**) DLC1 expression level influences the growth of MCF-7 cells. Cells were treated with control or DLC1 siRNA for 48 h, and treated with E2 for 72 h. (**H**) DLC1-mediated potentiation of ER transactivation depends on DLC1 status in MCF-7 cells. Cells were treated with control or DLC1 siRNA for 48 h. Cells were transfected with ERE-luc and treated with E2 for 24 h, after which ERE-luc activity was measured.

ER to the *pS2* gene chromatin. Interestingly, DLC1 overexpression in MCF-7/DLC1 (Dox-treated) cells, as well as oestrogen stimulation of the cells, led to significant enhancement of the ER interaction with the *pS2* gene chromatin (Fig 5A). When the above ChIP studies were repeated with T7 antibody to isolate T7-DLC1, we observed that DLC1 was efficiently recruited to *pS2* gene chromatin but that such recruitment was induced by oestrogen treatment (Fig 5B). Because oestrogen upregulated DLC1 in ER-positive cells, and because DLC1 might act as a chaperone for ER, our finding of the recruitment of the DLC1–ER complex to



pS2 gene chromatin suggests that DLC1 upregulation by oestrogen could potentially enhance ER transactivation, presumably through enhanced DLC1–ER interaction and its recruitment to the target gene chromatin. Because DLC1 does not have an obvious DNA-binding motif, we speculate that the binding of DLC1 to the target chromatin is through ER. To test this directly, we selectively knocked down the expression of ER by siRNA and found a significant reduction in the recruitment of DLC1 to *pS2* gene chromatin (Fig 5C), which suggests that DLC1 does not bind directly to the *pS2* promoter but rather does so through ER.

As DLC1 is a motor complex component with retrograde transport function and because DLC1 has been also found to interact with nuclear transcription factors in fission yeast (Kaiser *et al*, 2003), our findings suggested that the noted DLC1–ER interaction might be involved in the nuclear transport of ER. To investigate this possibility, we examined the subcellular localization of DLC1 and ER in Ishikawa cells, with or without knockdown of DLC1 expression, using confocal scanning microscopy. We found that DLC1 was located predominantly in the cytoplasm; however, we repeatedly observed a modest but easily detectable level of DLC1 in the nucleus (Fig 5D, left panel). Interestingly, downregulation of DLC1 levels by siRNA was accompanied by a reduction in the nuclear ER in more than 90% of cells (Fig 5D), suggesting that DLC1 acts as a chaperone for the nuclear translocation of ER. As previous studies have shown that DLC1 could function in both dynein motor-dependent and dynein motor-independent ways (Puthalakath *et al*, 1999), we explored whether ER also interacts with the dynein intermediate light chain (DIC, a marker of the dynein motor) in a DLC1-dependent or DLC1-independent manner. To test this, we immunoprecipitated DIC from cells treated with or without oestrogen. We found that ER also interacts with the dynein motor and such interaction was enhanced after oestrogen stimulation (Fig 5E). Next, we examined the effect of DLC1 knockdown on the ER–DIC interaction. We found that abolition of DLC1 resulted in a substantial inhibition of ER binding to DIC (Fig 5F), which suggests a potential role of DLC1 in the observed ER–DLC1 interaction with DIC.

In summary, we found that DLC1 has a facilitating role in the nuclear accumulation as well as in the transactivation function of

◀ **Fig 4** | DLC1 interacts with oestrogen receptor *in vitro* and *in vivo*. (**A**) Interaction of DLC1 with ER *in vitro*. GST pull-down assay shows the association of GST–DLC1 with *in vitro*-translated ^{35}S-labelled ER. (**B**) Interaction of DLC1 with the AB domain of ER. GST pull-down assay shows the association of GST–ER deletion constructs with the *in vitro*-translated ^{35}S-labelled DLC1. (**C**) Mapping the ER-binding region in DLC1. GST pull-down assay shows the association of GST–DLC1 deletion constructs with the *in vitro*-translated ^{35}S-labelled ER. (**D**) DLC1 and ER interact *in vivo*. Cell lysates were immunoprecipitated with anti-ER monoclonal antibody and blotted with anti-DLC1 or anti-ER antibodies. (**E**) DLC1 overexpression increases DLC1–ER interaction. MCF-7/DLC1 cells were treated or not treated with Dox and were stimulated with E2 for 1 h. Cell lysates were immunoprecipitated with anti-ER monoclonal antibody and blotted with anti-T7 or anti-ER antibodies. (**F**) ERE-luc assay shows that the deletion of the binding site of ER on DLC1 blocked transactivation.

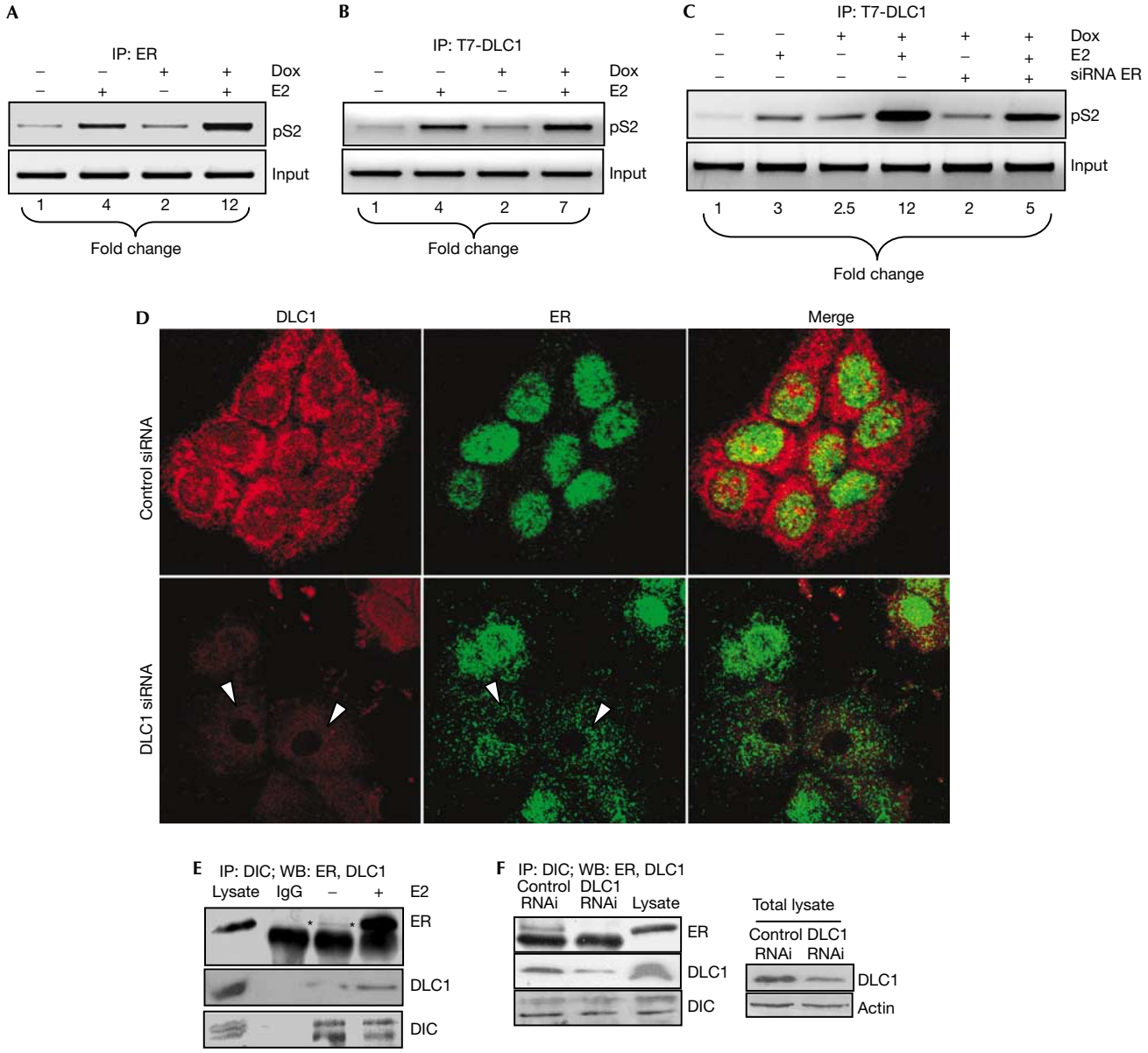


Fig 5 | DLC1 and oestrogen receptor localization and chromatin studies. (**A–C**) Recruitment of ER and DLC1 to the *pS2* promoter chromatin. MCF-7/DLC1 cells were treated or not treated with Dox for 24 h, stimulated with E2 for 1 h and ChIP was performed with anti-ER or anti-T7 antibodies. IP, immunoprecipitation. (**A**) DLC1 enhances ER recruitment. Upper panel: PCR analysis of the 304-bp *pS2* promoter fragment associated with ER. Lower panel: PCR analysis of the input DNA. Relative recruitment of ER or T7-DLC1 on the target promoter is shown as fold change. (**B**) Oestrogen induces recruitment of DLC1 to the target chromatin. PCR analysis of the 304-bp *pS2* promoter fragment associated with T7-DLC1 (upper panel); PCR analysis of the input DNA is shown below. (**C**) DLC1 recruitment to the *pS2* promoter chromatin depends on the presence of ER (upper panel). PCR analysis of the 304-bp *pS2* promoter fragment associated with T7-DLC1; PCR analysis of the input DNA is shown below. (**D**) Ishikawa cells grown on coverslips and treated with control or DLC1 siRNA for 48 h. The coverslips were incubated with antibodies against DLC1 and ER and then incubated with secondary antibodies conjugated with Alexa-546 (red) and Alexa-488 (green), respectively. Arrows indicate the cells with exclusively cytoplasmic staining of ER. (**E**) ER interacts with DIC *in vivo*. Cell lysates from MCF-7 stimulated with E2 for 1 h were immunoprecipitated with anti-DIC monoclonal antibody and blotted with anti-DLC1, anti-ER or anti-DIC antibodies. WB, western blot. (**F**) Role of DLC1 in ER–DLC1 interaction with DIC. Lysates from MCF7 cells were treated with control or DLC1 siRNA and were immunoprecipitated with anti-DIC monoclonal antibody and blotted with DLC1, ER and DIC antibodies. The right panel shows the extent of DLC1 knockdown by siRNA.

ER in breast cancer cells. Our observations that DLC1 interacted directly with ER and that oestrogen upregulation of DLC1 promoted its interaction with ER are important, because these findings suggest that both the level of DLC1 and ER activation status influence ER functions in breast cancer cells. Further, the DLC1–ER interaction has a role in the regulation of ER-inducible

genes and mitogenic responses, as the DLC1–ER complex was effectively recruited to ER target the *pS2* promoter chromatin. This finding, in turn, prompted us to determine the physiological significance of these findings and subsequently to delineate the chaperone-like function of DLC1 in conjunction with DIC in the potential nuclear accumulation of ER. Because the levels of DLC1 were increased by oestrogen, it is conceivable that putative upregulation of DLC1 could contribute to the ER-initiated cellular responses. Collectively, these findings suggest an important role for DLC1 in the action of ER and show a new mechanistic role for DLC1 in supporting and amplifying ER-initiated cellular responses in breast cancer cells.

METHODS

Constructs and generation of stable cell lines. We purchased the BAC clone (RPCI 11 18c24) containing the DLC1 genomic region from BACPAC Resources (Children's Hospital Oakland Research Institute, CA, USA). Details of constructs and generation of stable cell lines are provided in the supplementary information online.

Cell growth and soft agar assays. Growth assays were performed using standard methods as detailed in the supplementary information online.

Short interference RNA. DLC-specific siRNA (M-001000-00-50), DIC-specific siRNA (M-019799-00-0010) and control nonspecific siRNA (D-001206-13-20) were purchased from Dharmacon (Lafayette, CO, USA) and used as detailed in the supplementary information online.

Chromatin immunoprecipitation and confocal studies. Quantitative ChIP and confocal assays were performed as described previously (Vadlamudi *et al*, 2004), and are outlined in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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