Corrections

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The authors wish to note, “The legend of Fig. 2B should state that the experiment was performed in HMEC cells, rather than in MCF7 cells, similar to the confirmatory experiment presented in Fig. 2C.” The figure and its corrected legend appear below.

Fig. 2. Rapid induction of interchromosomal interactions by nuclear hormone signaling. (A) 3D-FISH confirmation of E2-induced (60 min) TFF1:GREB1 interchromosomal interactions in HMECs with the distribution of loci distances measured (box plot with scatter plot) and quantification of colocalization (bar graph) before and after E2 treatment. Cells exhibiting mono- or biallelic interactions were combined for comparison with cells showing no colocalization; statistical significance in the bar graph was determined by χ2 test (**, P < 0.001). (B) 2D FISH confirmation of the interchromosomal interactions in HMEC cells by combining chromosome paint (aqua) and specific DNA probes (green and red). (Upper) Illustrates two examples of mock-treated cells. (Lower) Shows the biallelic interactions/nuclear reorganization after E2 treatment for 60 min, exhibiting kissing events between chromosome 21 and chromosome 2. (C) Similar analysis on HMECs, but in this case using 3D FISH to paint chromosome 2 (red) and chromosome 21 (green), showing E2-induced chromosome 2–chromosome 21 interaction. Both assays revealed neither chromosome 21–chromosome 21 nor chromosome 2–chromosome 2 interactions in response to E2. (D) Temporal kinetics of GREB1:TFF1 interactions by 3D FISH in HMECs (**, P < 0.001 by χ2). (E–G) Nuclear microinjection of siRNA against ERα, CBP/p300, or SRC1/pCIP prevented E2-induced interchromosomal interactions, counting both mono- and biallelic interactions (**, P < 0.001 by χ2). The injection of siER and siDLC1 were done in the same experiment, sharing the same control group. (H) Nuclear microinjection of siRNA against LSD1, which was shown to be required for estrogen-induced gene expression (22), did not block E2-induced interchromosomal interactions. The injection of siLSD1 and SRC1/pCIP were done in a single experiment, sharing the same control group.

www.pnas.org/cgi/doi/10.1073/pnas.1400054111
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The authors wish to note: “We recently published corrections to two articles that describe the functional role (1) and enzymology (2) of caspase-12. The figures in these two articles have been thoroughly investigated by a committee at McGill University. With regard to Fig. 6 in the article in PNAS, two findings were determined: First, that republication of the data was a consequence of miscommunication among co-authors working in different locations, and second, that the molecular weight markers were unintentionally mislabeled. The latter issue has recently been corrected (3). The former issue has recently been rectified because figure 4 of the Nature article was replaced with a de novo independent experiment (4). Interpretation of the experiment and the conclusions of both the PNAS article and the Nature article are unaffected by these changes. The authors apologize for any confusion.”


www.pnas.org/cgi/doi/10.1073/pnas.1323789111

IMMUNOLOGY


The authors note that the accession number for the GEO database is GSE51393.

www.pnas.org/cgi/doi/10.1073/pnas.1400120111

MEDICAL SCIENCES, ENGINEERING


The authors note that the following statement should be added to the Acknowledgments: “This work is in part supported by Department of Defense (DoD) Breast Cancer Research Innovator Award W81XWH-10-1-0016 (to R.K.J.).”

www.pnas.org/cgi/doi/10.1073/pnas.1400494111
Editorial Expression of Concern and Correction

BIOCHEMISTRY

PNAS is publishing an Editorial Expression of Concern regarding the following article: “Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules,” by Qidong Hu, Young-Soo Kwon, Esperanza Nunez, Maria Dafne Cardamone, Kasey R. Hutt, Kenneth A. Ohgi, Ivan Garcia-Bassets, David W. Rose, Christopher K. Glass, Michael G. Rosenfeld, and Xiang-Dong Fu, which appeared in issue 49, December 9, 2008, of Proc Natl Acad Sci USA (105:19199–19204; first published December 3, 2008; 10.1073/pnas.0810634105).

The editors note that in January 2014, upon the authors’ request, PNAS published a correction to three figures in this article. Subsequent to the publication of the correction, the editors received concerns regarding images appearing in two of the corrected figures (Fig. 2B and Fig. S2) when compared with the data in the retracted Cell paper (1). In this regard, the authors note that the images in both the corrected Fig. 2B and Fig. S2 correspond to specific cells saved from large, multi-nuclei-containing photographic images. The authors note that this image-to-cell correspondence indicates that the incorrect cells shown in the retracted Cell paper resulted from errors in image processing. However, the original microscopy data is no longer available to further verify the accuracy of the images in question.

Inder M. Verma
Editor-in-Chief


www.pnas.org/cgi/doi/10.1073/pnas.1415649111

NEUROSCIENCE


The authors note that the following statement should be added as a new Acknowledgments section: “We thank Erin Light, Ashley Knutson, Kristin Mauldin, John Wixted, and Flora Suh for assistance. This work was supported by the Medical Research Service of the Department of Veteran Affairs, the National Institute of Mental Health Grant MH24600, and National Science Foundation (NSF) Grant SMA-1041755 to the Temporal Dynamics of Learning Center, an NSF Science of Learning Center. We also acknowledge the University of Bern face images database and face images from the Massachusetts Institute of Technology and the Center for Biological and Computational Learning.”

www.pnas.org/cgi/doi/10.1073/pnas.1415194111

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Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules

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Although the role of liganded nuclear receptors in mediating coactivator/corepressor exchange is well-established, little is known about the potential regulation of chromosomal organization in the 3-dimensional space of the nucleus in achieving integrated transcriptional responses to diverse signaling events. Here, we report that ligand induces rapid interchromosomal interactions among specific subsets of estrogen receptor α-bound transcription units, with a dramatic reorganization of nuclear territories, which depends on the actions of nuclear actin/myosin-I machinery and dynein light chain 1. The histone lysine demethylase, LSD1, is required for these ligand-induced interactive loci to associate with distinct interchromatin granules, long thought to serve as “storage” sites for the splicing machinery, some critical transcription elongation factors, and various chromatin remodeling complexes. We demonstrate that this 2-step nuclear rearrangement is essential for achieving enhanced, coordinated transcription of nuclear receptor target genes.

Results
Identification of Estrogen-Induced Interchromosomal Interactions.
We devised an initial, open-ended approach to detecting long-distance genomic interactions by coupling the chromosome conformation capture (3C) assay (21) with the ChIP-DSL strategy that we recently developed for large-scale promoter array and tiling array analyses (22), a method we refer to as deconvolution of DNA interaction by DSL or the 3D assay (Fig. 1A). In pilot experiments on MCF7 cells treated with E2 for 60 min, we isolated DNA after in situ restriction digestion followed by ligation under an extreme dilution condition according to the established 3C protocol, and after sonication of the DNA, we used a biotinylated oligonucleotides to capture DNA fragments that contain the enhancer of the well-studied E2-regulated TFF1 gene. To detect DNA fragments that were linked to the TFF1 enhancer during 3C, we annealed a set of DSL oligonucleotide pairs targeting individual genomic blocks in a ∼1.4 Mb tiled path surrounding the TFF1 gene in chromosome 21 (Fig. S1A). After selection, ligation, amplification, and hybridization on the corresponding tiling array according to the DSL protocol, we identified a series of specific intrachromosomal interactions that frequently involve other ERα-bound genomic loci, which were confirmed by the conventional 3C assay (Fig. S1B).

We included in the 3D experiment a set of tiled intervals on 6 different chromosomes, one of which encompassed GREB1, a well-characterized ERα-inducible gene located in chromosome 2 (22, 23). Based on selection of TFF1 interactants, 3D capture revealed 2 clusters of significant signals coincident with an enhancer and promoter in the GREB1 gene (Fig. 1B), whereas the other 5 tiled genomic regions on other chromosomes showed no signals, two of which are illustrated (Fig. 1B). This finding suggested E2-inducible intrachromosomal interactions between TFF1 and GREB1, which was validated by the conventional 3C assay (Fig. 1C).

To ensure that the interaction between the TFF1 and GREB1 genes, which reside in chromosome 21 and chromosome 2, respectively, occurred in both tumor and normal cells, we performed FISH analysis (24) on MCF7 and primary cultures of human mammary epithelial cells (HMECs), using a standardized


The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0810634105/DCSupplemental.

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protocol, including 4 days under serum deprivation and temporal synchronization of interphase nuclei. In each case approximately half of the cells exhibited monoallelic interactions, whereas the other half exhibited biallelic interactions (Fig. 2A), with the precise percentage differing for different lots of MCF7 cells or HMECs. Monoallelic interactions appear prevalent in other studies of interchromosomal interactions (14, 20, 25, 26). As controls, Dio1 and Casp7 loci exhibited no E2-induced interaction with TFF1 above FISH assay background (Fig. S2A).

To determine whether the detected interchromosomal interactions resulted only from long-distance DNA looping or were also accompanied by chromosomal movements/interactions, we performed chromosome painting in the presence or absence of FISH, using TFF1 and GREB1 probes, finding that, although chromosome 21 and chromosome 2 were independently localized in the nucleus before E2 treatment, the two chromosomes became intimately associated in many cells after hormone treatment (Fig. 2 B and C), suggesting the possibility that estrogen treatment exerted dramatic effects on nuclear architecture. Although we reproducibly detected >5-fold difference in chromosome 21–chromosome 2 pairing between mock-treated and E2-treated cells, the frequency of interacting chromosomal movement, in addition to long-distance DNA looping,
occurs in response to E2. Interestingly, neither chromosome 21–chromosome 21 nor chromosome 2–chromosome 2 interactions were observed in response to E2. A time-course with FISH analysis further revealed that the interchromosomal interactions had already occurred at the initial time point determined (15 min) after E2 treatment (Fig. 2D). These results establish rapid nuclear reorganization in interphase cells in response to ligand.

Requirements for ERα-Dependent Interchromosomal Interactions. Interactions between TFF1 and GREB1 were apparently dependent on ERα binding, because a specific siRNA, proven to effectively knockdown ERα (5, 27), effectively blocked the E2-induced GREB1:TFF1 interactions (Fig. 2E). We next performed single cell nuclear microinjection, using specific siRNAs or blocking antibodies against several specific coactivators, including CBP/p300 (Fig. 2F) and the p160 coactivator SRC1/pCIP (Fig. 2G), as established (5, 27), finding that inactivation of these coactivators for ERα also abolished the E2-dependent TFF1:GREB1 interactions. We also examined the histone lysine demethylase 1 (LSD1), which was recently shown to be essential for E2-dependent gene activation (5). Unexpectedly, we observed that LSD1 knockdown had little effect on the E2-dependent interchromosomal interactions between the two genes (Fig. 2H). This finding implies that E2-induced interchromosomal interactions precede gene activation.

Although there is no filamentous actin in the nucleus, nuclear actin is present in many transcriptional complexes and reported to play an important role in transcriptional activation in yeast (28, 29). We could also detect oligomerized g-actin with a specific monoclonal antibody (30) in the nucleus of normal breast epithelial cells (Fig. S3A). Treatment of E2-stimulated breast epithelial cells with either latrunculin, a well-characterized drug that blocks actin polymerization (31), or Jasplakinolide, which inhibits depolymerization of actin networks (32), caused a complete loss of E2-induced interchromosomal interactions (Fig. 3A and Fig. S2B). These treatments also inhibited E2-induced expression of TFF1 and GREB1, but not constitutive genes (e.g., β-actin) in MCF7 cells (Fig. 3B).

To further investigate the possibility that interchromosomal interactions depend on dynamic nuclear reorganization, we performed single cell nuclear microinjection assay to determine the potential requirement for Nuclear Myosin-I (NMI) (33), finding that a specific siRNA against NMI abolished E2-induced TFF1:GREB1 interactions (Fig. 3C). We also found that nuclear injection of antibodies against NMI blocked E2-induced TFF1:GREB1 interactions (Fig. 3C Right and Fig. S3 B–D), as did similar single cell nuclear microinjection of the monoclonal antibody (2G2) against g-actin (Fig. S3 B–D). Immunohistochemical analysis confirmed nuclear localization of injected IgG against NMI and g-actin (Fig. S3 B and D), strongly suggesting a direct functional requirement for NMI in the nucleus, rather than an indirect effect caused by a disrupted cytoskeleton. To document the involvement of NMI-based nuclear motors in mediating E2-induced interchromosomal interactions, we performed rescue experiments, using NMI mutants that impair actin binding (e.g., R353C) or the ATPase activity (e.g., S397L) in the nuclear myosin-I “head” (34, 35). We found that the interchromosomal interactions abolished with anti-NMI IgG could be restored using the expression vector expressing WT, but not mutant NMI defective in actin binding or lacking ATPase activity (Fig. 3 D and E). This finding agrees with a recent report showing that NMI is required for transcription activation-induced DNA looping out of the nuclear territory (36). We also documented that WT, but not functionally inactive NMI mutants, rescued the expression of TFF1 (Fig. 3F) and GREB1 (data not shown) after knockdown of endogenous NMI in MCF7.

Based on a recent report that the dynein light chain-1 (DLC1) directly interacts with liganded ERα (37), we examined the effect of DLC1 inactivation by siRNA or injection of a blocking antibody, both of which effectively abolished GREB1:TFF1 interactions in E2-treated primary breast epithelial cells (Fig. 4A).
The components of the nuclear motor system act after initial as CBP/p300, to the genes indicated, were quantified by RT-qPCR. Mean treated with siRNAs against the chromatin remodeling factor consistent with the multifactorial orchestration of interchromosomal interactions. Disruption of nuclear microinjection of siRNA or antibody against 19202 and Fig. S5); similar data were observed with siRNA against the chromatin remodeling factor BAF53 (Fig. 4 B–D), consistent with the multifactorial orchestration of interchromosomal interactions. Disruption of NMI or DLC1 by siRNA did not cause aberrant recruitment of ERα or its coactivators, such as CBP/p300, to the TFF1 promoter (Fig. S6), which suggests that these components of the nuclear motor system act after initial ERα binding and coactivator recruitment events.

**Functional Consequences of Induced Interchromosomal Interactions.**

Both the siRNA and the antibody microinjection experiments described above established a correlation between hormone-induced interchromosomal interactions and gene activation. To directly demonstrate the functional requirement of E2-induced interchromosomal interactions for gene activation, we took advantage of the observation that ~50% of cells exhibited monoallelic interactions between chromosome 2 and chromosome 21 to ask whether the transcription of gene loci engaged in the interchromosomal interactions was enhanced compared with noninteracting alleles in the same cells. We performed RNA FISH using probes that span exon-exon junctions and confirmed the specificity of the probes in detecting RNA by showing that RNase A treatment (14) abolished hybridization signals (data not shown). By determining the volume of the RNA signals for each transcript for colocalted and noncolocalized signals, we were able to quantify the level of transcription associated with interacting and noninteracting alleles before and after the E2 treatment (Fig. 4E). We found that E2 induced modest activation of TFF1 and GREB1 from “noninteracting” alleles, suggesting that both alleles may be equally competent in transcriptional activation. Remarkably, expression of these ERα target genes exhibited enhancement when engaged in specific interchromosomal interactions, therefore demonstrating the functional significance of interchromosomal interactions in enhancement of gene activation; indeed, this was actually more robust in cells with monoallelic, as opposed to biallelic, interactions (Fig. 4E). Three color DNA:RNA serial FISH experiments, using a TFF1 DNA FISH probe confirmed the colocalization of the genomic locus, and the two colocalted RNA transcripts (data not shown).

**Interchromatin Granules: Hubs for Interchromosomal Interactions?**

Having established nuclear actin/myosin-mediated gene networking in the nucleus, we next determined potential nuclear domains that permit or underlie such functional interchromosomal interactions. We suspected a possible spatial relationship with nuclear speckles, formally known as interchromatin granules that are enriched with several key transcriptional elongation factors, chromatin remodeling complexes, and essentially all factors required for pre-mRNA splicing (7, 38). To test this hypothesis, we determined colocalization between FISH probes and the splicing factor SC35, a marker for interchromatin granules (39). In mock-treated primary breast epithelial cells, the position of the TFF1 and GREB1 foci were entirely distinct from SC35-positive speckles. Upon E2 treatment, however, the interacting TFF1/GREB1 foci became intimately associated with two of the SC35-positive speckles. Intriguingly, in cells exhibiting monoallelic interactions, the interacting loci, but not noninteracting loci, were observed to associate with interchromatin granules (Fig. 5A). In cells with biallelic interactions, both were present in interchromatin granules (Fig. 5B). Blocking actin oligomerization with latrunculin or actin depolymerization with cytochalasin impaired the association (Fig. S5C). Likewise, siRNAs against DLC1 or BAF53 all similarly blocked the colocalization of the FISH probes with interchromatin granules (Fig. S5 B and C and data not shown). These findings suggest that interchromatin granules may function as hubs for gene networking in the nucleus.

Because LSD1 siRNA was able to block E2-dependent transcription of TFF1 and GREB1, but not their interchromosomal interactions, we investigated whether there might be an effect on their coalescence with nuclear speckles. Intriguingly, depletion of LSD1 by specific siRNA prevented the TFF1/GREB1 loci from interacting with interchromatin granules as determined by both 2D and 3D FISH with wild-type, but not enzymatically inactive LSD1, fully rescuing hub: interchromatin granule interaction (Fig. 5C–E). In concert with our previous observation that LSD1 siRNA decreased, but did not abolish ERα recruitment (5), we detected only a modest reduction in the recruitment of coactivators, such as CBP/p300, to the TFF1 target genes in response to E2 (Fig. S7). Together, these findings reveal an unexpected role of LSD1 in exerting a key regulatory function in linking transcriptional initiation to full gene activation by promoting the association of initial interacting loci to nuclear domains enriched with critical factors for transcription and cotranscriptional processing.

**Discussion**

Our findings reveal a previously unappreciated role of liganded nuclear receptors in initiating specific interchromosomal inter-
actions. The formation of such gene networks now proves to be of functional importance for ligand-dependent enhancement of gene transcription. Our data also connect nuclear receptor-mediated recruitment of coactivators to the actions of the nuclear motor machinery in establishing ligand-induced intra- and interchromosomal interactions and the association of interacting gene loci with interchromatin granules, which constitutes a more complex program for hormone-induced gene expression than previously suspected. Our finding that NMI plays a key role in E2-induced gene activation program is consistent with its role in coordinated and enhanced regulation of gene expression by permitting efficient coupling of transcriptional initiation, elongation, and RNA processing events.

Materials and Methods

Detailed protocols for cell culture, signal cell nuclear microinjection, and pharmacological treatment of cells are described in the SI Methods.

ChIP-DSL, 3D, and 3C assays. Genomic tiling by ChIP-DSL was described (5, 22). Two anti-ERα antibodies (HC-20 and H-184; Santa Cruz Biotechnology) were combined for ChIP analyses. The 3D assay began with the conventional 3C procedure, restriction digestion with BamHI and BglIII, using the procedure identical to that described for mammalian cells (52). Details of these assays are presented in the SI Methods. Oligonucleotides used for 3C validation are listed in Table S1.

DNA and RNA FISH. The cells were processed for FISH essentially as described in ref. 24 except that oligonucleotide probes labeled with specific hapten were used as listed in Table S2. Both 2D and 3D FISH were performed. For triple-labeled FISH, probes to promoter regions were labeled at the 5′ position with digoxigenin (DIG) and probes to enhancer regions were labeled with either Biotin (Bio) or Fluorescein (FITC). For double-labeled FISH, promoters were labeled with Bio and enhancers with FITC. After hybridization, specific probes were detected by using a mix of quantum dot (Q-dot)-conjugated antibodies in 1:200 dilution (sheep anti-DIG Fab fragment primary antibody-conjugated with Q-dot 655, streptavidin-conjugated with Q-dot 605, and goat anti-FITC whole IgG primary antibody-conjugated with Q-dot 525, all from Invitrogen). For a complete list of antibodies used in this study, see Table S3. Q-dots were mildly sonicated before use.

Single chromosome paint probes were commercially acquired from Applied Spectral Imaging (Vista). Each probe was custom-labeled with different fluorophores: chromosome 1 (1-585-649) in aqua, red and green, respectively. Hybridization and detection protocols were performed as recommended by the manufacturer. Data acquisition and analysis are described in the SI Methods.

RNA FISH was performed with modification of published techniques (CSH...
Protocols; 2007–prot4763). All reagents were RNase-free. Cells were prepared as described in ref. 24 with the addition of a dehydration step by means of an ethanol series to 100% ethanol. Sequence of the specific oligo probes used is provided in Table S2. Slides were washed and signal was detected with Q-dot antibodies as previously described. Slides were treated with RNase A before signal detection as a control.

ACKNOWLEDGMENTS. We thank V. Malhotra and S. Emr for insightful suggestions, R. Piha-Jarzynka for help in performing initial DNA FISH to independently confirm E2-induced interchromosomal interactions, J. Feramisco and C. Murre for invaluable assistance in microscopy studies, Troy Ideker for advice on statistical analysis, Begem Lee for technical assistance, C. Nelson for cell culture, M-J. Jin and L-X. Duan of Aviva Systems Biology for assistance in performing ChIP-DSL and 3D assays, J. Hightower and M. Fisher for assistance with figure and manuscript preparation, and S. Cattaert of Santa Cruz Biotechnology for advice on antibody reagents. O.H. was supported by the Cancer Research Institute; M.D.C. was supported by an American-Italian Cancer Foundation Postdoctoral Research Fellowship. M.G.R. is a Howard Hughes Medical Institute Investigator. This work was supported by National Institutes of Health Grants CA52599 and HL8129 (to G.K.C.), GM043569 and CA114184 (to X.-D.F.), and CA97134, DK39949, N0304394 HL65445, DK018477; DOD W81XWH-07-PCRP-IDA and W81XWH-08-1-0665, PFC (to M.G.R.).


10. Heng FC, Lee H, Zhang WX, Yen YH, Lin YS, Khinchanet and D. Spector for expert help in performing initial DNA FISH to independently confirm E2-induced interchromosomal interactions, J. Feramisco and C. Murre for invaluable assistance in microscopy studies, Troy Ideker for advice on statistical analysis, Begem Lee for technical assistance, C. Nelson for cell culture, M-J. Jin and L-X. Duan of Aviva Systems Biology for assistance in performing ChIP-DSL and 3D assays, J. Hightower and M. Fisher for assistance with figure and manuscript preparation, and S. Cattaert of Santa Cruz Biotechnology for advice on antibody reagents. O.H. was supported by the Cancer Research Institute; M.D.C. was supported by an American-Italian Cancer Foundation Postdoctoral Research Fellowship. M.G.R. is a Howard Hughes Medical Institute Investigator. This work was supported by National Institutes of Health Grants CA52599 and HL8129 (to G.K.C.), GM043569 and CA114184 (to X.-D.F.), and CA97134, DK39949, N0304394 HL65445, DK018477; DOD W81XWH-07-PCRP-IDA and W81XWH-08-1-0665, PFC (to M.G.R.).