



**DNA Oxidation as Triggered by H3K9me2
Demethylation Drives Estrogen-Induced Gene
Expression**

Bruno Perillo, *et al.*
Science **319**, 202 (2008);
DOI: 10.1126/science.1147674

***The following resources related to this article are available online at
www.sciencemag.org (this information is current as of January 11, 2008):***

Updated information and services, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/cgi/content/full/319/5860/202>

Supporting Online Material can be found at:

<http://www.sciencemag.org/cgi/content/full/319/5860/202/DC1>

This article **cites 25 articles**, 10 of which can be accessed for free:

<http://www.sciencemag.org/cgi/content/full/319/5860/202#otherarticles>

This article appears in the following **subject collections**:

Molecular Biology

http://www.sciencemag.org/cgi/collection/molec_biol

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:

<http://www.sciencemag.org/about/permissions.dtl>

grafts after viral reactivation (Fig. 4A). This CD4⁺ T cell proliferation was significantly reduced after transplantation into MHC II-deficient recipients as compared with such proliferation in B6 controls, implying a further role for blood-recruited APCs in local T cell activation (Fig. 4B). Unexpectedly, these MHC II-deficient recipients also showed attenuated expansion of DRG-resident gB-specific T cells (Fig. 4C), arguing that the local memory CD8⁺ T cell activation was itself CD4⁺ helper T cell-dependent. To confirm this finding, we grafted latent ganglia, taken from donor mice depleted of CD4⁺ cells just before harvest, into recombination-activating gene (RAG)-deficient mice. Eliminating the CD4⁺ T cells inhibited CD8⁺ T cell expansion on secondary antigen encounter (Fig. 4D), although it did not compromise their intrinsic responsiveness because the cells were still able to produce interferon- γ after *in vitro* stimulation (fig. S6). The tissue-resident CD4⁺ T cells alone were sufficient to elicit help because the RAG-deficient recipients are otherwise devoid of T cells. It should be noted that primary HSV-specific CD8⁺ T cell activation is also helper T cell-dependent (24).

At face value, our results appear to contradict findings arguing that antigen encounter within nonlymphoid tissues does not result in local T cell proliferation (6, 7). However, it may prove that such a lack of proliferation is only seen under certain conditions: for example, during activation of the T cells by parenchymal cells or where the

virus is rapidly brought under control by resident T cells (25). As a consequence, we propose that local responses are tailored to the level of inflammation or the extent of infection. In settings with limited inflammation or where presentation of virus antigen is confined to the parenchyma, pure elaboration of effector function results in little overall T cell expansion. If, however, infection progresses and DC recruitment comes into play, then local T cells proliferate *in situ*. Finally, our results provide insight into HSV immune evasion during reactivation. Virus-specific CD8⁺ T cells, thought capable of suppressing reactivation (12, 26), are lost from latent ganglia in situations that promote virus recrudescence (16). The natural delay in DC infiltration and local T cell expansion affords a natural opportunity for transient HSV replication before the virus is once again brought under renewed immune control.

References and Notes

- U. H. von Andrian, C. R. Mackay, *N. Engl. J. Med.* **343**, 1020 (2000).
- R. L. Reinhardt, A. Khoruts, R. Merica, T. Zell, M. K. Jenkins, *Nature* **410**, 101 (2001).
- D. Masopust, V. Vezys, A. L. Marzo, L. Lefrançois, *Science* **291**, 2413 (2001).
- H. Hikono *et al.*, *Immunol. Rev.* **211**, 119 (2006).
- N. van Panhuys, R. Perret, M. Prout, F. Ronchese, G. Le Gros, *Trends Immunol.* **26**, 242 (2005).
- K. H. Ely *et al.*, *J. Immunol.* **170**, 1423 (2003).
- N. L. Harris, V. Watt, F. Ronchese, G. Le Gros, *J. Exp. Med.* **195**, 317 (2002).
- J. E. Moyron-Quiroz *et al.*, *Immunity* **25**, 643 (2006).

- D. J. Zammit, L. S. Cauley, Q. M. Pham, L. Lefrançois, *Immunity* **22**, 561 (2005).
- J. Banchereau, R. M. Steinman, *Nature* **392**, 245 (1998).
- G. J. Randolph, K. Inaba, D. F. Robbiani, R. M. Steinman, W. A. Muller, *Immunity* **11**, 753 (1999).
- T. Liu, K. M. Khanna, X. Chen, D. J. Fink, R. L. Hendricks, *J. Exp. Med.* **191**, 1459 (2000).
- A. L. van Lint *et al.*, *J. Virol.* **79**, 14843 (2005).
- Materials and methods are available as supporting material on *Science* Online.
- J. G. Stevens, M. L. Cook, *Science* **173**, 843 (1971).
- M. L. Freeman, B. S. Sheridan, R. H. Bonneau, R. L. Hendricks, *J. Immunol.* **179**, 322 (2007).
- R. B. Tenser, K. A. Hay, W. A. Edris, *J. Virol.* **63**, 2861 (1989).
- F. Tacke, G. J. Randolph, *Immunobiology* **211**, 609 (2006).
- B. Leon, M. Lopez-Bravo, C. Ardavin, *Immunity* **26**, 519 (2007).
- S. L. Bailey, B. Schreiner, E. J. McMahon, S. D. Miller, *Nat. Immunol.* **8**, 172 (2007).
- F. Ginhoux *et al.*, *Nat. Immunol.* **7**, 265 (2006).
- F. Tacke *et al.*, *J. Exp. Med.* **203**, 583 (2006).
- M. Le Borgne *et al.*, *Immunity* **24**, 191 (2006).
- S. R. Jennings, R. H. Bonneau, P. M. Smith, R. M. Wolcott, R. Chervenak, *Cell. Immunol.* **133**, 234 (1991).
- R. J. Hogan *et al.*, *J. Exp. Med.* **193**, 981 (2001).
- K. M. Khanna, R. H. Bonneau, P. R. Kinchington, R. L. Hendricks, *Immunity* **18**, 593 (2003).
- The authors thank R. Allan, L. Eidsmo, T. Gebhardt, K. Shortman, J. Villadangos, W. Halford, and B. Gebhardt for helpful discussions. This work was supported by the Australian National Health and Medical Research Council and the Howard Hughes Medical Institute.

Supporting Online Material

www.sciencemag.org/cgi/content/full/319/5860/198/DC1

Materials and Methods

Figs. S1 to S6

References

17 October 2007; accepted 16 November 2007

10.1126/science.1151869

DNA Oxidation as Triggered by H3K9me2 Demethylation Drives Estrogen-Induced Gene Expression

Bruno Perillo,^{1*} Maria Neve Ombra,^{1*} Alessandra Bertoni,² Concetta Cuozzo,³ Silvana Sacchetti,³ Annarita Sasso,² Lorenzo Chiariotti,² Antonio Malorni,¹ Ciro Abbondanza,⁴ Enrico V. Avvedimento^{2†}

Modifications at the N-terminal tails of nucleosomal histones are required for efficient transcription *in vivo*. We analyzed how H3 histone methylation and demethylation control expression of estrogen-responsive genes and show that a DNA-bound estrogen receptor directs transcription by participating in bending chromatin to contact the RNA polymerase II recruited to the promoter. This process is driven by receptor-targeted demethylation of H3 lysine 9 at both enhancer and promoter sites and is achieved by activation of resident LSD1 demethylase. Localized demethylation produces hydrogen peroxide, which modifies the surrounding DNA and recruits 8-oxoguanine-DNA glycosylase 1 and topoisomerase II β , triggering chromatin and DNA conformational changes that are essential for estrogen-induced transcription. Our data show a strategy that uses controlled DNA damage and repair to guide productive transcription.

Estrogens control growth and survival of hormone-sensitive cells by inducing expression of genes important for cell cycle progression (1) and apoptosis (2). The hormone 17 β -estradiol (E2) regulates transcription of target genes by binding to cognate estrogen receptors (ER α and ER β), which then bind with high affinity to estrogen-responsive DNA elements (EREs)

(3–5). After hormone activation, ER α interacts with transcription co-activators that covalently modify histone proteins within nucleosomes (6) and contribute to the control of gene expression (7).

We examined the molecular mechanism by which specific methylation and demethylation of lysines in histone H3 governs hormone-induced transcription. We studied the anti-apoptotic *bcl-2*

gene that contains two EREs in the coding region (2) and validated our findings with the prototypic E2-target gene *pS2* (4, 5). With chromatin immunoprecipitation (ChIP) analysis, we assessed DNA binding of estrogen receptors in ER α ⁺ human breast cancer MCF-7 cells (8). Figure 1A shows that DNA fragments from the *bcl-2* ERE enhancer region (enh.) and the upstream promoter (pr.), which are 1.5 kb apart (9), are bound by ER α after treatment of quiescent cells with 10 nM E2 for 30 min.

Binding of both regions that reside on different fragments in sonicated DNA suggests loop formation between the *bcl-2* promoter and the ERE enhancer. To test this hypothesis, we used the chromosome conformation capture (3C) technique, which detects transcription-dependent chromatin looping caused by dynamic interactions between distant DNA segments. Briefly, after cross-linking of proteins with formaldehyde, we ligated restriction-digested DNA ends: DNA circles were generated by ligation of sites not contiguous on linear DNA (10). Figure 1B shows that the *bcl-2* promoter and enhancer were bridged (when co-amplified with divergent primers) only in chromatin harboring activated ER α (45 min of E2). The promoter-enhancer loop (or ligated DNA circle in the 3C) was lost by 60 min of E2 addition. Moreover, the receptor was essential in this process because DNA bending was inhibited by treatment with the antiestrogen ICI 182,780 that prevents receptor binding to the DNA (Fig. 1B).

Assembly of productive ER α transcription complex between 30 and 45 min (fig. S1) was marked by a substantial increase of dimethyl-Lys⁴ in histone H3 (H3K4me2) on promoter and enhancer regions of *bcl-2* or *pS2* gene, whereas presence of H3K9me2 was appreciably found in hormone-starved cells or after 60 min of E2 treatment, concurrent with loss of receptor from chromatin (Fig. 1C and fig. S1). Binding of the K4-specific histone methyl transferase (HMT) SET9 (11) and the K9-specific HMT Suv39H1 (12) peaked at 30 min and 60 min of E2 exposure, respectively (Fig. 1C).

We show that H3K9me2 demethylation at the promoter and enhancer sites is achieved by the lysine-specific demethylase LSD1, known to relieve transcriptional repression by demethylating K9me and K9me2 (13). In fact, LSD1 was found to be present on chromatin in both the presence and the absence of estrogens (Fig. 2A), and disappearance of K9me2 was prevented by treatment of cells with the monoamine oxidase inhibitor pargyline (13) or by LSD1 knockdown with specific small interfering RNAs (siRNAs) (Fig. 2A).

LSD1 is also responsible for demethylation of H3K4me2 (14) observed 60 min after E2 addition and in hormone-starved cells, and this step is mediated by ER α (Fig. 2, B and C). In fact, in the ER α MDA-231 breast cancer cells and in fibroblasts, H3K4 and H3K9 are both dimethylated at these sites (Fig. 2C), as also observed for surrounding chromatin in MCF-7 cells (fig. S2). Expression of the receptor in quiescent MDA-231 cells resulted in specific loss of H3K4me2 (Fig. 2C). Therefore, in the absence of hormone, ER α contacts and transiently activates LSD1, leading to local H3K4 demethylation. However, under these conditions, LSD1 is unable to trigger demethylation of H3K9me2 and transcription initiation. In fact, a transcriptionally unproductive cycle has been described, solely driven by the receptor (5).

Local demethylation of H3K9me2 was triggered by recruitment of liganded ER α to chromatin (15) that was also required for RNA polymerase II (pol II) assembly on the promoter (Fig. 2D). Inhibition of LSD1 activity in the presence of hormone prevented chromatin looping (Fig. 2E) and estrogen-dependent transcription (Fig. 2F), even though the receptor was bound to the EREs (Fig. 2A).

Demethylation by LSD1 of H3K9me2 is an oxidative process that results in the production of hydrogen peroxide (H₂O₂) (16). To explore the

possibility that formation of H₂O₂ is a signal induced by estrogens to drive transcription initiation, we measured the main DNA product induced by the peroxide: the 8-oxo-guanine (8-oxo-G) that is removed by base excision repair (BER) enzymes (17). As shown in Fig. 3A, estrogens caused a rapid nuclear accumulation of 8-oxo-Gs in discrete foci, mimicked by treatment with

H₂O₂ and inhibited by scavengers of reactive oxygen species (ROS), such as N-acetylcysteine (NAC). Production of 8-oxo-Gs was tightly linked to LSD1 activation (hence demethylation of H3K9me2) because it was inhibited by pargyline or LSD1 knockdown (Fig. 3, A to C). Furthermore, oxidation of Gs was dependent on ER α because the fluorescent signal was detected in

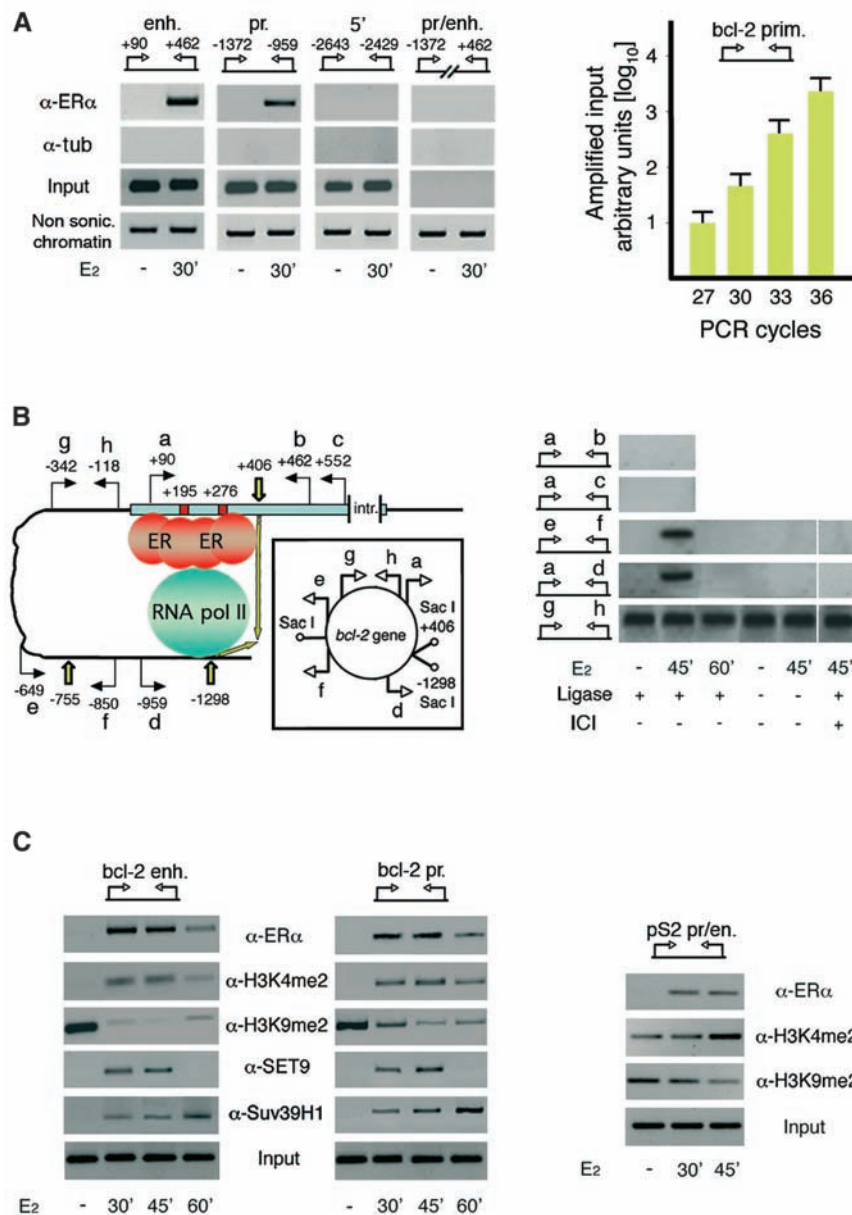


Fig. 1. Dynamics of estrogen-dependent chromatin modifications. **(A)** (Left) ChIP experiments performed with antibodies to ER α (α -ER α) or α -tubulin (α -tub) and with primers spanning the *bcl-2* gene. Primers are numbered relative to the translation start site (+1). enh., enhancer; pr., promoter; 5', upstream DNA. pr/enh., DNA including promoter and enhancer. Input, DNA extracted from sonicated (sonic.) chromatin before immunoprecipitation and used to quantify PCR reactions at different cycles and with different primer (prim.) pairs (right). Error bars indicate SEM. **(B)** (Left) Simplified structure of *bcl-2* gene showing the boxed coding region with EREs (red). intr., intron. Sac I restriction sites (yellow arrows) and primers used for PCRs in 3C experiments (thin arrows) are reported. The square box includes the circle generated by DNA ligation in the 3C. (Right) PCRs performed with divergent (a to d) or convergent (e and f) primers. The a-d (655 bp) or the e-f (202 bp) bands were absent in amplifications realized on Sac I-digested naked DNA. **(C)** Kinetic ChIP experiments realized on cells stimulated for 30, 45, and 60 min with 10 nM E2.

¹Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche (C.N.R.), 83100 Avellino, Italy. ²Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano," Università degli Studi "Federico II," 80131 Naples, Italy. ³Naples Oncogenomic Center, Centro di Ingegneria Genetica (CEINGE), Biotecnologie Avanzate, 80131 Naples, Italy. ⁴Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, 80138 Naples, Italy.

*These authors contributed equally to this paper. †To whom correspondence should be addressed. E-mail: perillo@unina.it (B.P.); awedim@unina.it (E.V.A.)

Fig. 2. Histone H3K9me2 demethylation by LSD1 is essential for DNA bending induced by activated ER α . **(A)** ChIP experiments on *bcl-2* and *pS2* promoters with indicated antibodies in cells exposed to E2 in the absence or presence of pargyline or control scrambled (scram.) RNAs or siRNAs targeting LSD1 coding region (LSD1-1). **(B)** Effect of pargyline addition on H3K4me2 demethylation, assessed by ChIP. **(C)** ChIPs showing presence of H3K4me2 and K9me2 in MDA-231 cells, expressing (ER α ⁺) or not (wild type, WT) ER α , or in human primary fibroblasts. **(D)** Effect of prevention of ER α binding to DNA by 1 μ M ICI on LSD1 activation and RNA pol II recruitment to *bcl-2* promoter. **(E)** 3C assessing the effect of LSD1 inhibition on DNA loop formation. Primers used in PCRs are the same as in Fig. 1B. **(F)** Transcription of *bcl-2* and *pS2* genes measured by nuclear run on in cells stimulated with E2. β -actin was used as hormone-unresponsive control.

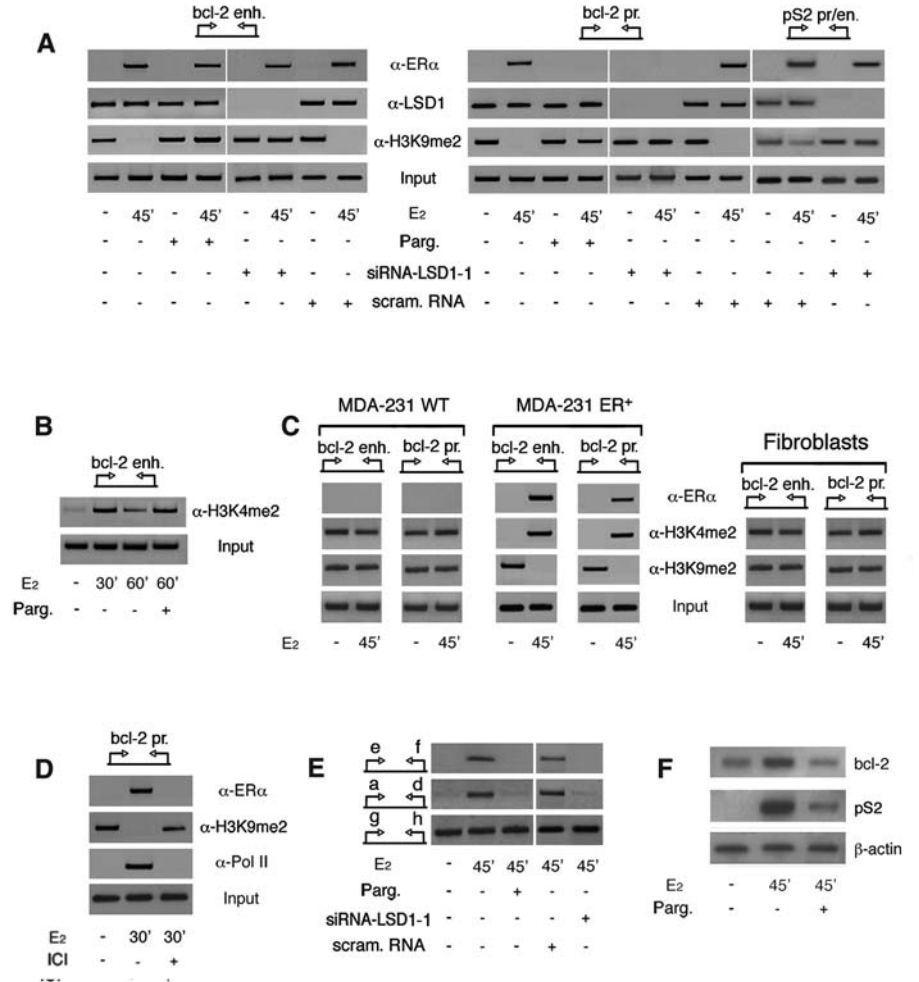
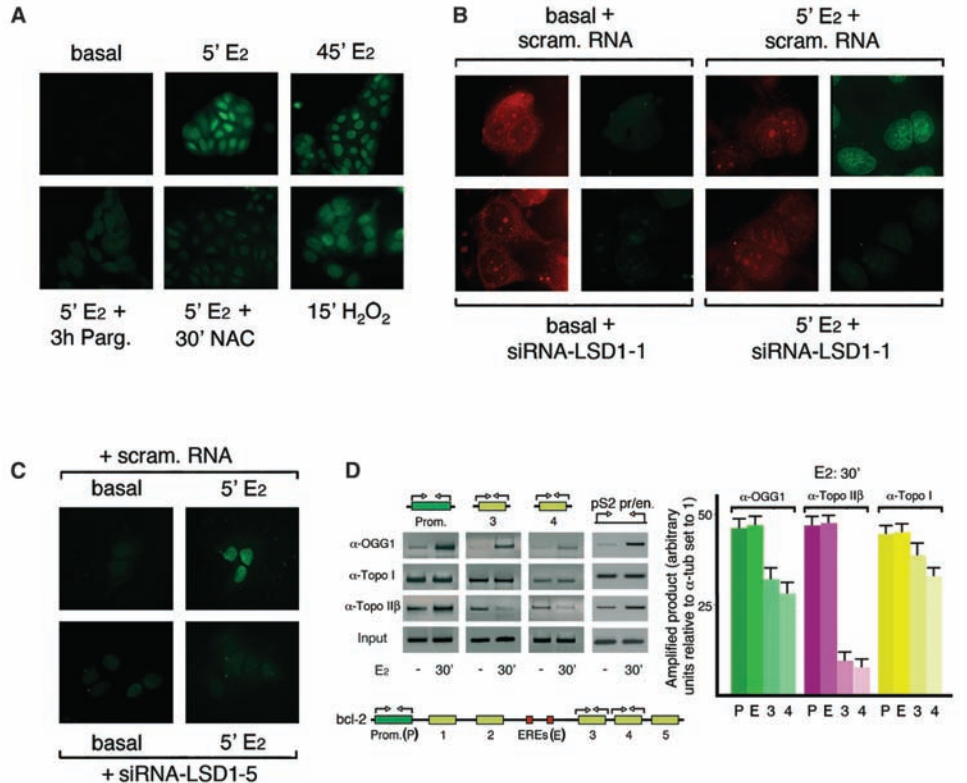


Fig. 3. Estrogens induce a burst of nuclear 8-oxo-Gs and recruitment of OGG1 and topoisomerase II β (Topo II β) to the promoter and ERE region of E2-responsive genes. **(A)** and **(C)** Immunofluorescence detection of 8-oxo-Gs by the fluorescein-tagged 8-oxo-G-binding protein. **(A)** Effect of pargyline or NAC addition to hormone-exposed cells. **(B)** and **(C)** Assessment of LSD1 knockdown, with two different siRNAs targeting the coding region (LSD1-1 and LSD1-5), on accumulation of 8-oxo-Gs. Scrambled (scram.) RNA was used as control. Red fluorescent protein (DsRed1-N1) was added to visualize transfected cells. **(D)** ChIP assays with reported antibodies. Topo I, topoisomerase I. The lower supplement shows the location of *bcl-2* DNA regions used in ChIP analysis (indicated by arrows pairs) The histogram on the right is from four different experiments. A representative gel with the *pS2* promoter is also shown. Error bars indicate SEM.



cells with NAC that did not prevent H3K9me2 demethylation (Fig. 4C) inhibited chromatin bending (Fig. 4D) and, hence, inhibited accumulation of active polymerase on promoter (Fig. 4E) and transcription (fig. S6). On the other hand, H₂O₂, besides enhancing per se recruitment of phosphorylated RNA pol II, also restored its E2-dependent promoter assembly in cells with pargyline added (Fig. 4F). Therefore, ER α plays a pivotal role in directing the oxidative signal to the promoter. Subsequently, OGG1 and topoisomerase II β (but with different efficiencies) are recruited to the promoter after peroxide accumulation; in the absence of hormone, however, their targeting is dispersed throughout chromatin, without the selective gradient imposed by bound receptor: H₂O₂ does not substitute for ER α (fig. S6).

Estrogens induce high OGG1 protein levels, and this induction is prevented by NAC or mimicked by H₂O₂ (Fig. 4G). Moreover, the proteasome inhibitor MG132 similarly induces high OGG1 levels (Fig. 4G). Because the proteasome catalytic subunit LMP2 assembles with the ER α complex and is essential for estrogen-induced transcription (24), we suggest that the oxidative burst, initiated by H3K9me2 demethylation, inhibits the proteasome activity and increases the intracellular concentration of OGG1. Thereafter, extinction of the demethylation-ROS wave leads to proteasome

reactivation and degradation-release of the receptor (60 min after E2 stimulation) (Fig. 1C). Production of H₂O₂ is temporally limited and strictly localized (25).

H3K9me2 demethylation at the promoter and enhancer regions selectively marks the site where transcription initiation complex is assembled to drive the cycle of estrogen-induced gene expression (movie S1). Hence, the RNA pol II complex intimately associates with DNA repair enzymes to initiate hormone-dependent gene expression. Local lysine demethylation, with its associated oxidative burst, may indeed represent the initial signal at other inducible promoters.

References and Notes

1. S. F. Doisneau-Sixou *et al.*, *Endocr. Relat. Cancer* **10**, 179 (2003).
2. B. Perillo, A. Sasso, C. Abbondanza, G. Palumbo, *Mol. Cell. Biol.* **20**, 2890 (2000).
3. D. J. Mangelsdorf *et al.*, *Cell* **83**, 835 (1995).
4. Y. Shang, X. Hu, J. DiRenzo, M. A. Lazar, M. Brown, *Cell* **103**, 843 (2000).
5. R. Metivier *et al.*, *Cell* **115**, 751 (2003).
6. B. D. Strahl, C. D. Allis, *Nature* **403**, 41 (2000).
7. T. Jenuwein, C. D. Allis, *Science* **293**, 1074 (2001).
8. M. Shanmugam *et al.*, *Mol. Cell. Endocrinol.* **148**, 109 (1999).
9. R. L. Young, S. J. Korsmeyer, *Mol. Cell. Biol.* **13**, 3686 (1993).
10. A. Ansari, M. Hampsey, *Genes Dev.* **19**, 2969 (2005).
11. H. Wang *et al.*, *Mol. Cell* **8**, 1207 (2001).

12. O. Vaute, E. Nicolas, L. Vandel, D. Tronche, *Nucleic Acids Res.* **30**, 475 (2002).
13. E. Metzger *et al.*, *Nature* **437**, 436 (2005).
14. Y. Shi *et al.*, *Cell* **119**, 941 (2004).
15. I. Garcia-Bassets *et al.*, *Cell* **128**, 505 (2007).
16. F. Forneris, C. Binda, M. A. Vanoni, A. Mattevi, E. Battaglioli, *FEBS Lett.* **579**, 2203 (2005).
17. A. P. Grollman, M. Moriya, *Trends Genet.* **9**, 246 (1993).
18. N. Rajapakse, M. Butterworth, A. Kortenkamp, *Environ. Mol. Mutagen.* **45**, 397 (2005).
19. T. Roldan-Arjona *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8016 (1997).
20. B.-G. Ju *et al.*, *Science* **312**, 1798 (2006).
21. M. T. Russo *et al.*, *Cancer Res.* **64**, 4411 (2004).
22. S. S. David, V. L. O'Shea, S. Kundu, *Nature* **447**, 941 (2007).
23. P. E. Kroeger, N. Osheroff, T. C. Rowe, *J. Biol. Chem.* **268**, 16449 (1993).
24. H. Zhang *et al.*, *EMBO J.* **25**, 4223 (2006).
25. Q. Felty *et al.*, *Biochemistry* **44**, 6900 (2005).
26. This work has been supported by the Ministero dell'Università e delle Ricerche (MUIR), RBNE0157EH, by Associazione Italiana per la Ricerca sul Cancro (AIRC), and by Federazione Italiana per la Ricerca sul Cancro (FIRC), NOGEC (Naples Oncogenomic Center), CEINGE. We are grateful to the memory of G. Salvatore, S. Varrone, and J. Guardiola.

Supporting Online Material

www.sciencemag.org/cgi/content/full/319/5860/202/DC1
Materials and Methods
Figs. S1 to S6

11 July 2007; accepted 19 November 2007
10.1126/science.1147674

Designed Protein-Protein Association

Dirk Grueninger, Nora Treiber, Mathias O. P. Ziegler, Jochen W. A. Koetter, Monika-Sarah Schulz, Georg E. Schulz*

The analysis of natural contact interfaces between protein subunits and between proteins has disclosed some general rules governing their association. We have applied these rules to produce a number of novel assemblies, demonstrating that a given protein can be engineered to form contacts at various points of its surface. Symmetry plays an important role because it defines the multiplicity of a designed contact and therefore the number of required mutations. Some of the proteins needed only a single side-chain alteration in order to associate to a higher-order complex. The mobility of the buried side chains has to be taken into account. Four assemblies have been structurally elucidated. Comparisons between the designed contacts and the results will provide useful guidelines for the development of future architectures.

In a cell, permanently associated proteins guarantee mechanical integrity, whereas transient associations are indispensable for metabolism and the regulation thereof. Our study focused on permanent contacts, many of which have been established at atomic resolution (1). Extensive analyses (2–8) showed that these contacts are diversified and that only a few rather general rules can be extracted: The contact area should be larger than about 600 Å², and the contacting surfaces should be complementary and

predominantly nonpolar. Moreover, the contribution of hydrogen bonds and salt bridges at the contact rim is negligible. It is rather easy to destroy a contact by introducing a bulky side chain that is sterically incompatible. It is also comparatively simple to produce weak contacts randomly, as demonstrated by many examples using surface mutations to induce proteins to crystallize (9, 10). However, the creation of a desired permanent contact is a more difficult task (11, 12). Whereas establishing tight contacts by screening is well known from the antigen-antibody system that has also been applied in engineered complexes (13), the designed production of a tight novel contact has to our knowledge only been tried once, but the system failed to crystallize (14). However, in a number of cases, a given

contact was modified by mutations (15–18). Here, we report the production and analysis of designed permanent *homo* oligomers of five proteins.

The importance of symmetry for the association of identical protein subunits was recognized some time ago (19). At the lowest symmetry level, a single contact patch has to provide the whole binding energy, which usually involves a large number of residues. As a consequence, the creation of such a contact requires numerous mutations. Because these contacts also generally form infinite helices that cannot be crystallized (Fig. 1A), we did not try to construct such an assembly. In contrast, a C₂-symmetric dimer (20) has a contact multiplicity of 2 and therefore requires only half the number of mutations. Moreover, it is usually globular and thus crystallizable (Fig. 1A). The same applies to higher symmetries. Associating two C₂-symmetric dimers along their molecular axes results in a D₂ tetramer with a contact multiplicity of 4 (20), whereas C₄ tetramer units give rise to D₄ octamers with a multiplicity of 8 (fig. S4). Obviously, the higher the symmetry, the fewer mutations are required, which explains the abundance of symmetry in natural *homo* oligomers.

Whereas the symmetry concerns the framework of a design, the actual production of *homo* oligomers was based on the exchange of side chains on the surface. We refrained from touching any main chains, because the corresponding structural changes are much more difficult to predict. Because contacts rigidify the involved

Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität, Albertstrasse 21, 79104 Freiburg im Breisgau, Germany.

*To whom correspondence should be addressed. E-mail: georg.schulz@ocbc.uni-freiburg.de