CCR frontiers IN SCIENCE

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STRUCTURAL BIOLOGY

Protein-Protein Interactions: What Are the Preferred Ways in Which Proteins Interact?

Ma B, Elkayam T, Wolfson H, and Nussinov R. Protein-protein interactions: structurally conserved residues distinguish between binding sites and exposed protein surfaces. *Proc Natl Acad Sci U S A* 100: 5772–7, 2003.

What are the preferred ways in which proteins interact? The understanding and the ability to predict the preferred mode of protein associations can provide tremendous insight into molecular and cellular biology. Protein structures can associate in different ways. Mapping protein interactions on a genomic scale is essential for effective drug design. Protein-protein interactions are crucial to the understanding of practically all *in vivo* functions—cellular regulation, biosynthetic and degradation pathways, signal transduction, initiation of DNA replication, transcription and translation, multimolecular associations, packaging, the immune response, and oligomer formation. However, despite the broad recognition of the importance of deciphering the complex nature of protein interactions, they are still not entirely understood.

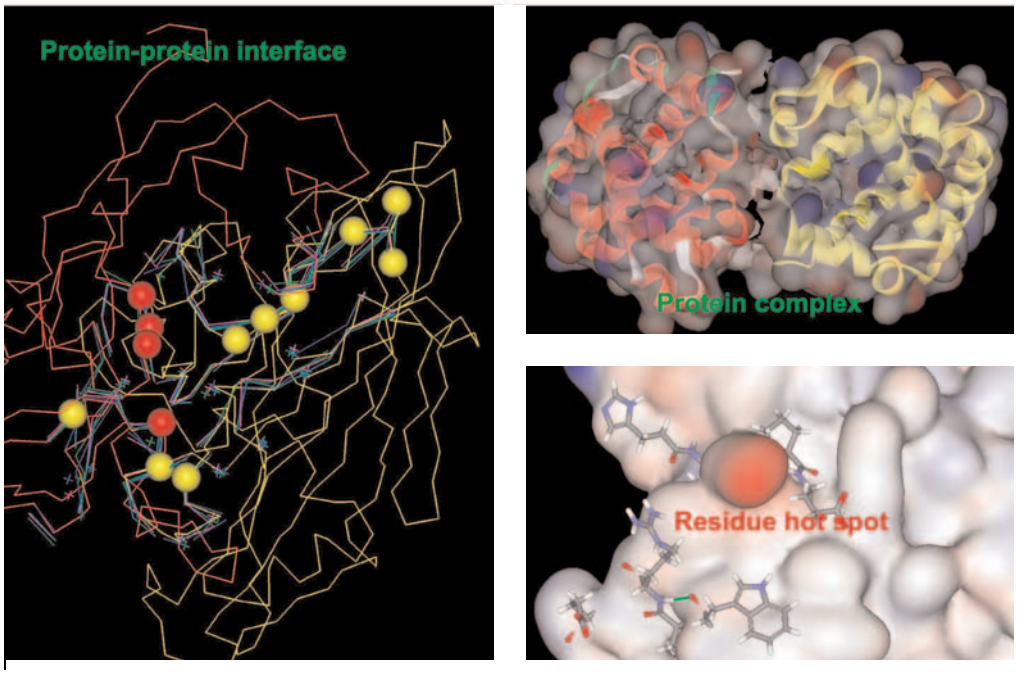

Figure 1. The left-hand side illustrates an interface between two protein molecules. Only the backbone chains are shown. One protein chain is in red, the other in yellow. The residue hot spots (red and yellow balls, in the corresponding chains) are conserved in homologous interfaces. The homologous interfaces are shown in different colors (light blue, dark blue, green, and violet) superimposed on the red and yellow chains. Top, right-hand side: The entire protein-protein complex, in a space-filled representation. The backbones are highlighted as ribbons. Bottom, right-hand side: A residue hot spot (red ball) and the surrounding residues in the protein-protein interface.

Assisted by our novel, amino acid sequence, order-independent multiple structural comparison algorithms (Shatsky M et al. *Proteins* 56: 143–56, 2004), we have been able to derive a structural dataset of protein-protein interfaces (Keskin O et al. *Protein Science* 13: 1043–55, 2004) to superimpose the multiple members of the interface clusters and obtain conserved residues (Ma B et al. *Proc Natl Acad Sci U S A* 100: 5772–7, 2003). We have observed that conserved residues correlate with the residue hot spots identified by alanine-scanning mutagenesis (DeLano WL. *Curr Opin Struct Biol* 12: 14–20, 2002). Hot spots are residues that, when mutated to alanine, lead to an increase in the absolute binding energy by more than 2 kcal/mol (Bogan AA and Thorn KS. *J Mol Biol* 280: 1–9, 1998). We have further observed that the hot spots occur predominantly at the interfaces of macromolecular complexes, distinguishing protein binding sites from the remainder of the surface. Consequently, hot spots can be used to define binding epitopes—areas on proteins to which ligands bind.

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In particular, conservation of tryptophan on the protein surface indicates that it is highly likely that there is a binding site at this location. To a lesser extent, conservation of phenylalanine and methionine also implies a binding site. Figure 1 illustrates a protein-protein interface, a complex, and a hot spot residue.

We have investigated the organization of the hot spots and the structurally conserved residues (Keskin O et al. *J Mol Biol* 345: 1281–94, 2005). Interestingly, the hot spots are clustered within locally, highly packed regions, explaining the conservation of these residues and their large stabilizing contributions. Within the dense clusters, they form a network of interactions, and consequently, their contributions to the stability of the complex are *cooperative*. However, the contributions of independent clusters are additive. This suggests that the binding free energy is not a simple summation of the single hot spot residue contributions and highlights the similarity between binding and folding where conserved residues occur in folding nuclei.

Furthermore, this hot spot organization reconciles the apparent conflicting observations: On the one hand, electrostatic interactions are well known to be extremely important in protein associations (Sheinerman FB et al. *Curr Opin Struct Biol* 10: 153–9, 2000), yet on the other hand, we observed charge-charge conserved residue couples to be underrepresented (Halperin I et al. *Structure [Camb]* 12: 1027–38, 2004). The densely packed regions effectively screen the electrostatic interactions between the conserved charged hot spot residues. Thus, packing—a major player in folding—has a crucial role in binding. Conserved polar residues at the binding interfaces confer rigidity to minimize the entropic cost of binding, whereas the surrounding residues form a flexible cushion. Hence, the picture that emerges is that protein-protein associations are optimized locally, with the clustered, networked, highly packed structurally conserved residues contributing dominantly and cooperatively to the stability of the complex. When addressing the crucial question of "what are the preferred ways in which proteins interact," these findings point toward a critical involvement of *hot regions* in protein-protein interactions.

Comparisons of different crystal packing interfaces or of mutant variants in the crystallographic database illustrate that similar protein structures can associate in different ways. However, the clustered protein-protein interface dataset further illustrates that proteins that have different functions, different sequences, and different global structures can associate in similar ways. It is intriguing that proteins, regardless of their family origins and functions, have preferred organizations. Binding and folding are thus similar processes, refined by evolution for function.

Preferred organization is a key in chemistry and in protein science, whether in amyloid microfilaments or in globular protein-protein associations. Evolution re-utilizes preferred, favorable patterns and modulates these toward different functions. This highlights the potential use of the protein-protein interface dataset toward binding site prediction. Structural comparisons of the interfaces against protein structures in the structural database are likely to identify currently unknown sites and identify (or validate) proposed functions, pathways, and cellular networks. Combined with other existing cellular-pathway and protein-interaction databases, such approaches will integrate biology at different levels. Above all, we envision that observations such as those described here and those attained from future work along such integrative lines will provide insight into the answer to one of the most profound of questions: Can we *predict* the ways proteins will likely interact?

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Lsh, a Guardian of Heterochromatin

Yan Q, Cho E, Lockett S, and Muegge K. Association of Lsh, a regulator of DNA methylation, with pericentromeric heterochromatin is dependent on intact heterochromatin. *Mol Cell Biol* 23: 8416–28, 2003.

The genome is organized into densely packaged "silent" zones of heterochromatin and more loosely arranged zones of "active" euchromatin. Epigenetic modifications such as DNA methylation or specific histone tail modifications mark the different states of chromatin. Heterochromatin represses transcription and controls centromere function during mitosis. Defects in heterochromatin organization are responsible for a number of human inherited diseases and are thought to play a role in tumorigenesis.

Lsh is a member of the SNF2 family that uses ATP for nucleosomal remodeling. It is ubiquitously expressed during embryogenesis and is a crucial factor in normal murine development. We previously demonstrated that Lsh controls important characteristics of heterochromatin such as DNA methylation and histone tail modifications. In order to define the molecular function of Lsh, we addressed more recently the question of whether it participates directly in the configuration of heterochromatin (as opposed to indirectly inducing other protein factors) and determined some of the requirements for successful recruitment of Lsh to its targets.

As expected by its homology to chromatin-remodeling proteins, Lsh is exclusively detected in the nuclear fraction of cells. A series of Lsh deletional mutants demonstrated that the N-terminal portion of Lsh was indispensable for nuclear localization.

To examine the nuclear compartment of Lsh localization, we performed biochemical fractionation of cellular lysates. We found Lsh to be closely associated with chromatin, as has been reported for other SNF2 family members or DNA methyltransferases (but unlike most transcription factors or nuclear proteins, such as MECP2 and PCNA). Mutational analysis of the Lsh protein revealed that the internal and C-terminal regions of Lsh are required for this interaction with chromatin.

To determine further the nuclear structure to which Lsh bound, we examined green fluorescent protein (GFP)—tagged Lsh by confocal immunofluorescence analysis. Lsh was found to accumulate in spots and co-localized with stains for the DNA dye DAPI. This dye preferentially intercalates into AT-rich regions such as major satellite sequences that compose pericentromeric heterochromatin in murine cells. To further verify pericentric localization, we co-stained Lsh with antibodies that detect HP1α or tri-methylated H3-K9me, two important characteristics of pericentric heterochromatin. Furthermore, using antibodies against Lsh in chromatin immunoprecipitations (ChIPs) confirmed a direct association of Lsh with satellite sequences that are specific for pericentric heterochromatin.

Although Lsh co-localizes with Dnmt1 (the major maintenance DNA methyltransferase) during late replication, Lsh does not show a close functional interaction with Dnmt1. Lsh is also not crucial for recruitment of Dnmt1 to replication forks, considering that the distribution of Dnmt1 did not change in Lsh-deficient cells. Moreover, Lsh does not require Dnmt1 for its localization since Lsh is continually associated with pericentric heterochromatin throughout the cell cycle, whereas Dnmt1 accumulates at pericentric heterochromatin only at late S-phase.

Which signal is required for localization of Lsh to heterochromatin? DNA methylation appears not to be a crucial signal because transiently expressed GFP-tagged Lsh can still localize to pericentric heterochromatin in hypomethylated fibroblasts (such as $Lsh^{-/-}$ cells). Also, tri-methylation of H3-K9me and HP1α does not appear to be crucial for the targeting of Lsh to pericentric sites, considering that Lsh is still able to associate with pericentric heterochromatin in $Suv39h1/h2^{-/-}$ cells that lack H3-K9tri-me and normal HP1α distribution. In contrast,

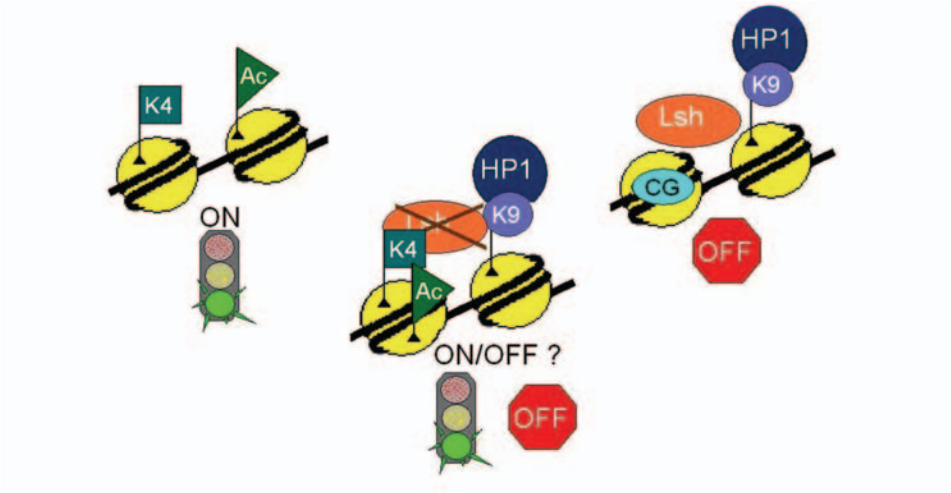

Figure 1. Lsh is directly associated with heterochromatin. Transcriptional (active) chromatin is usually characterized by histone acetylation (Ac) and methylation at lysine 4 (K4) of histone 3 (H3) (left graphic). In contrast, silent heterochromatin at repeat elements is characterized by DNA methylation (CG), H3-K9me (K9), HP1 binding, and association of Lsh (right graphic). Lsh-deleted cells show perturbed heterochromatin with normal H3-K9me, but elevated H3-K4me and histone acetylation levels and a derepression of silent repeat elements (middle graphic).

trichostatin A (TSA) treatment that disrupts higher-order heterochromatin organization abrogates the signal for normal Lsh recruitment. Also, Lsh does not bind to chromatin nor does it accumulate at pericentromeric regions, suggesting that Lsh recruitment requires intact higher-order heterochromatin structure. However, the signal for Lsh recruitment appears to be reversible, because depletion of TSA leads to a recovery of higher-order heterochromatin structure and proper Lsh localization within 24 hours. This suggests that binding of Lsh to pericentromeric heterochromatin is dependent on histone modifications and closely associated with normal higher-order structure of heterochromatin.

Our results indicate that Lsh directly associates with heterochromatin (Figure 1) and, therefore, show an important link with previous findings that Lsh deletion leads to perturbed heterochromatin structure. Lsh-deficient cells show, for example, a global loss of CpG methylation of about 50% compared with the wild type and an increase in H3-K4me and histone acetylation levels at pericentric satellite sequences. These results support the idea that Lsh plays a direct role in the formation of heterochromatin (as opposed to indirectly enhancing the expression of other chromatin modifying components). Furthermore, the role of Lsh in pericentromeric heterochromatin formation may be crucial for normal centromeric function. Pericentric heterochromatin is thought to be critical for sister chromatid cohesion and chromosomal segregation during mitosis. Thus, the loss of Lsh at pericentric heterochromatin may be partly responsible for the mitotic defects that we observed in Lsh-deficient fibroblasts. Recent studies further extend our knowledge about genomic targets for Lsh. For instance, Huang J et al. found that Lsh deletion leads to epigenetic changes and derepression of transcription at many repetitive sequences beyond pericentric satellite sequences (*Nucleic Acids Res* 32: 5019–28, 2004). For example, Lsh controls the suppression of parasitic retroviral elements that are widely dispersed throughout the genome. Since Lsh is also directly associated with these repeat elements (as demonstrated by ChIPs analysis), the data suggest that Lsh plays a direct role in the formation at dispersed heterochromatic sites throughout the genome.

Taken together, these results support the model that Lsh acts as a guardian of normal heterochromatin structure. Revealing more about Lsh's molecular function should promote our understanding of the role of heterochromatin in diseases such as cancer.

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■ CELL BIOLOGY

Two Sequences Determine Where Replication Starts at the *β-Globin* Locus

Wang L, Lin CM, Brooks S, Cimbora D, Groudine M, and Aladjem MI. The human beta-globin replication initiation region consists of two modular independent replicators. *Mol Cell Biol* 24: 3373–86, 2004.

During each cell cycle, cells must duplicate their genetic material in order to transmit a complete copy of the genome to each daughter cell. Because of the large size of the human genome, cells use hundreds of thousands of replication starting points. DNA sequences that determine replication starting points are called "replicators." Replicators bind protein complexes that transmit instructions from the cell cycle machinery to the DNA duplex, ensuring that DNA replication will start only when the cells are ready. When DNA replication escapes cell cycle controls, the result is genomic instability, a hallmark of cancer cells.

Although replicators were identified in single-cell organisms more than two decades ago, mammalian replicators are not well understood. In previous studies, we examined a particular stretch of DNA, named IR (for initiation region), located within the human *β-globin* locus. Replication of this locus starts within IR. When we moved IR out of its normal location and inserted it into a genomic site where replication does not normally initiate, it was able to start DNA replication at the new location. These observations suggested that IR is a replicator. In the current study, we examined which parts of IR contained information that allowed replication.

We first looked for small fragments of the transferred IR that could start replication. Surprisingly, we found that IR actually contained two replicators, each capable of dictating where replication initiates (Figure 1). One of these replicators contains the promoter for the *β-globin* gene, while the other spans an intron. We termed these two replicators Rep-P (for promoter) and Rep-I (for intron). We then looked for deletions that destroyed IR's initiation activity, reasoning that the deleted sequences will contain essential information that allows initiation to occur.

We found that Rep-P and Rep-I each contained two sequences that were essential for replicator activity. One of these sequences was a stretch of adenines and thymines (AT-rich stretch),

which is often found in replication initiation sites. The AT-rich stretches were essential for starting DNA replication, but they could not, by themselves, determine where replication initiated. The second sequence exhibited an asymmetric structure: It contained mainly purines (adenines and guanines) on one strand and mainly pyrimidines (thymidines and cytosines) on the other strand. This sequence cooperates with the AT-rich stretch to start replication.

Until now, one of the difficulties in studying replication in mammalian cells was that researchers could not find a shared sequence common to all initiation sites. This limited our ability to decipher how cells recognize replicators. The observations reported in the featured paper suggest that replicators are composed of modules, and that these modules share common sequence features such as AT-richness and asymmetry. It is not clear if all the replicators in mammalian cells are recognized by the same features; however, identifying modules allows us to ask whether these sequences bind specific protein factors that transmit signals from the cell cycle machinery to chromatin.

An interesting aspect of the study is that the IR is actually a cluster of two replicators. For years, replication was thought to start from single sites termed "replication origins" in some genetic loci, and from broad, undefined "initiation zones" in others. Our findings blur this distinction and suggest that even well-defined, concise replication starting sites may contain more than one replicator. Why do cells require multiple replicators? The answer may lie in the need to coordinate replication with other processes that occur on chromatin. Replication requires the assembly of a large complex of proteins at the initiation site, which accumulates on chromatin early during the G1 phase of the cell cycle and may interfere with other DNA-protein interactions such as transcription complexes. Therefore, not all the potential replicators may be utilized in any given cell cycle. Indeed, initiation sites, and the timing of DNA replication, can change in different tissues, during development, or in response to specific environmental conditions. Clusters of potential replicators may provide flexibility and serve to ensure that replication will start at least at one point in each genomic locus. Future studies will use the protein binding patterns in Rep-I and Rep-P to determine how cells choose which replicator will start DNA synthesis during normal growth and under conditions that perturb the cell cycle control over DNA replication.

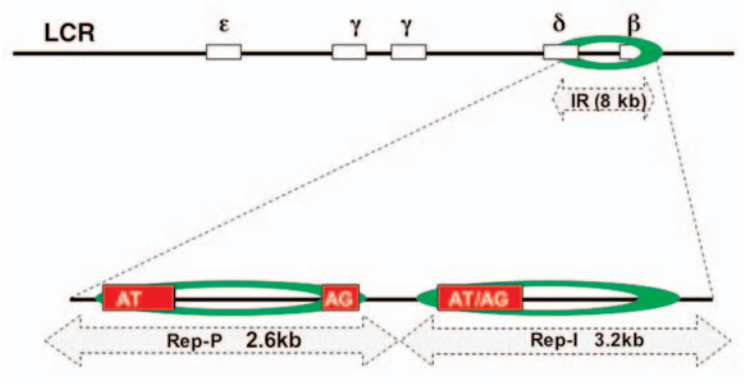

Figure 1. At a low resolution, the *β-globin* initiation region (IR) seemed to dictate replication at a single location, between the two adult genes encoding the β-globin protein (open rectangles). By genetic dissection, we learned that there are actually two replicators within the IR, each of which is capable of starting replication (green elipses). Once the duplication was taken into account, sequence elements that contribute to replicator ability (red) could be readily elucidated, and were shown to share common sequence features (AT tracts and asymmetric AG sequences). LCR, locus control region.

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New Required Intramural Acknowledgement in Publications Will Aid in Tracking of Intramural Contribution to Science

As of July 23, 2005, the NIH Office of Intramural Research requires that all intramural scientists include in the acknowledgements section of all of their publications the following statement:

> **"This research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research."**

The wording should be precisely as stated since it will be used to track the publications. Any divergence from this wording will hinder the tracking and lead to possible exclusion of the publication. The [in part] should be removed when the research was fully funded by intramural research.

NIH intramural research makes a large contribution to the world of science. However, that contribution is not always acknowledged or even known, while extramural contributions are, and have always been, carefully tracked.

Therefore, the reasons for this new requirement are two-fold: First, it will allow for tracking which publications come from intramural scientists despite variation in the way journals report addresses of co-authors. Second, this acknowledgement will highlight the important role that the intramural program plays in a great variety of innovative and collaborative research. As you know, scientists supported with extramural funds already are required to acknowledge NIH in their publications. Any questions should be directed to Tracy Thompson (thompstr@mail.nih.gov).

Suppression of EGFR and ErbB2 Induces Apoptosis in Aneuploid Cells

Pack S, Alper ÖM, Stromberg K, Augustus M, Özdemirli M, Miermont AM, Klus G, Rusin M, Slack R, Hacker NF, Ried T, Szallasi Z, and Alper Ö. Simultaneous suppression of epidermal growth factor receptor and C-ErbB-2 reverses aneuploidy and malignant phenotype of a human ovarian carcinoma cell line. *Cancer Res* 64: 789–94, 2004.

Epithelial ovarian cancer is the most lethal gynecological malignancy in the United States due to the fact that it is commonly diagnosed at an advanced stage. Like many other carcinomas, ovarian cancers are genetically unstable, and this instability results in an abnormal number of chromosomes, that is, "aneuploidy." This aneuploidy is accompanied by uncontrolled growth and reduced cell-to-cell, cell-to-matrix, and cell-to-basement membrane adhesiveness. A complex network of signaling pathways governs the growth of cancer cells and their metastatic potential. The critical role of cell surface growth factor receptors in cell growth, differentiation, and tumorigenesis is well established. For instance, two protein products of the *ErbB* supergene family, ErbB1, better known as epidermal growth factor receptor (EGFR) and ErbB2 (which shows structural homology with EGFR), have tyrosine kinase activity and are involved in the development of ovarian cancer.

We previously demonstrated that downregulation of EGFR possibly plays a role in growth inhibition of a human ovarian carcinoma cell line (NIH:OVCAR-8). First, we showed that blockade of the cAMP pathway and its associated serine-threonine kinase activity resulted in a decrease in ErbB tyrosine kinase activity in NIH:OVCAR-8 cells that overexpressed both EGFR and ErbB2 (Alper Ö et al. *Oncogene* 18: 4999–5004, 1999), and inhibition of either one of these receptors resulted in the growth arrest of additional ovarian cancer cell lines.

We further studied the effects of EGFR suppression on cell adhesion and tumorigenicity in NIH:OVCAR-8 cells (Alper Ö et al. *Int J Cancer* 88: 566–74, 2000). The inhibition of EGFR expression *in vitro* resulted in decreased levels of E-cadherin and α- and β-catenins. This resulted in the inhibition of cell-to-cell interactions. We then demonstrated in a murine tumor model that blockade of EGFR expression inhibits tumor formation in mice. The EGFR pathway is also involved in the control of metalloproteinase levels. These enzymes degrade the basement membrane or extracellular matrix, and this degradation facilitates invasion of tumor cells into surrounding tissues, a potential first step for the generation of local and distant metastasis. We could demonstrate that, in NIH:OVCAR-8 cells, reduction of EGFR levels caused a decrease in metalloproteinase secretion, and resulted in an inhibition of cancer cell migration and invasion (Alper Ö et al. *J Natl Cancer Inst* 93: 1375–84, 2001).

The reduction of EGFR expression, therefore, exerts inhibitory effects on growth, migration, and invasive potential of ovarian cancer cell lines. However, this inhibition is often temporary; one of the reasons for this might be found in the retained transforming ability of EGFR-inhibited ovarian tumor cells (unpublished observation based on soft-agar assays). This could be possible due to redundant signaling pathways: Blocking EGFR alone, therefore, might not be sufficient to prevent tumor recurrence and metastasis. NIH:OVCAR-8 cells overexpress both EGFR and ErbB2. We hypothesized that simultaneous targeting of both genes might result in a more complete and permanent growth inhibition. This hypothesis was supported by findings showing that reduction of ErbB2 expression resulted in a reduction of transforming potential in NIH:OVCAR-8 cells (Pegues JC and Stromberg K. *Cancer Lett* 117: 73–9, 1997).

We have now studied the consequences of the simultaneous inhibition of both EGFR and ErbB2 in NIH:OVCAR-8 cells. This was achieved by exposing the cells to both antisense RNAs and monoclonal antibodies. Dual blockade of EGFR and ErbB2 resulted in the inhibition of cancer cell invasiveness. The comparison of the karyotypes of the untreated and dual-treated cells using chromosome analysis and CGH (comparative genomic hybridization) also revealed a karyotype shift in the cell population: Aneuploid cells appeared more sensitive to this dual inhibition, which resulted in the preferential elimination of aneuploid clones, and the selection of near-diploid and diploid cells. This karyotype shift was accompanied by distinct gene expression changes: cDNA microarray analysis showed upregulation of tankyrase, fibronectin, and progesterone receptor mRNA expression levels. The phenotypic consequences of these gene expression changes were consistent with a reversal of the malignant phenotype such that gene expression signatures were more similar to those observed in normal ovarian cells. Our results, therefore, clearly demonstrate that simultaneous suppression of EGFR and ErbB2 reverses aneuploidy and the malignant phenotype of a human ovarian carcinoma cell line.

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Apoptotic Topoisomerase I–DNA Complexes Induced by Staurosporine and Arsenic Trioxide: A Point of No Return

Sordet O, Khan QA, and Pommier Y. Apoptotic topoisomerase I–DNA complexes induced by oxygen radicals and mitochondrial dysfunction. *Cell Cycle* 3: 1095–7, 2004.

DNA topoisomerase I (Top1) is ubiquitous and essential, as it relaxes DNA supercoiling ahead of replication and transcription complexes. DNA relaxation is attributable to the induction of Top1-associated single-strand breaks, thereby allowing rotation of the DNA double helix around the intact phosphodiester bonds opposite from the enzyme-mediated DNA cleavages. Once the DNA is relaxed, Top1 readily religates the breaks and regenerates intact duplex DNA. Under normal conditions, the covalent Top1-cleaved DNA intermediates, referred to as "cleavage complexes," are transients and remain at very low levels—the DNA religation ("closing") step being much faster than the DNA cleavage ("nicking") step.

The molecular mechanisms leading to enhanced Top1 cleavage complexes can be schematically divided in two groups (http://discover.nci.nih.gov/pommier/pommier.htm). First, Top1 cleavage complexes can be trapped by specific inhibitors such as camptothecins. These anticancer drugs specifically bind at the Top1-DNA interface and trap the cleavage complexes by preventing the DNA religation step. The second mechanism is related to frequent DNA lesions such as oxidized bases, abasic sites, mismatches, and strand breaks that interfere with Top1's religation reactions.

Recently, we discovered Top1 cleavage complexes in cells undergoing apoptosis. These "apoptotic Top1 cleavage complexes" were observed in different human cell lines exposed to arsenic trioxide (As_2O_3) (Sordet O et al. *J Biol Chem* 279: 33968–75, 2004) or staurosporine (Sordet O et al. *J Biol Chem* 279: 50499–504, 2004), two mechanistically different inducers of apoptosis. The heavy metal As_2O_3 and the alkaloid kinase inhibitor staurosporine are inactive on purified Top1, indicating that apoptotic Top1 complexes form indirectly in treated cells.

We next found that apoptotic Top1-DNA complexes are secondary to oxidative DNA lesions occurring during As_2O_3- and staurosporine-induced apoptosis as a result of reactive oxygen species (ROS) production. Indeed, modulation of ROS levels was directly correlated with the formation of Top1 cleavage complexes by As_2O_3 or staurosporine, suggesting that the ROS-dependent formation of oxidative DNA lesions is a common mechanism for the trapping of Top1 cleavage complexes during apoptosis. Accordingly, cellular exposure to H_2O_2 also induces Top1 cleavage complexes (Daroui P et al. *J Biol Chem* 279: 14587–94, 2004). Thus, we propose that As_2O_3 and staurosporine (as well as a wide range of apoptotic stimuli) induce the generation of reactive oxygen species (ROS) that damage DNA (oxidized bases, abasic sites), thereby generating Top1 cleavage complexes in apoptotic cells (Figure 1).

Mitochondria are likely to participate in the production of the ROS and the Top1 cleavage complexes during apoptosis, since Bcl-2 overexpression prevents the formation of the apoptotic Top1 cleavage complexes. Bcl-2 prevents the permeabilization of the outer mitochondrial membrane, and therefore the release of cytochrome C and the downstream activation of caspases. Activated caspase-3 feeds back on permeabilized mitochondria, which further dissipates the mitochondrial transmembrane potential ($\Delta\psi m$) and induces the further accumulation of intracellular ROS. Caspase activation is involved in the generation of the apoptotic cleavage complexes considering that z-VAD-fmk prevents As_2O_3- and staurosporine-induced ROS and Top1 cleavage complexes. Activation of caspases could therefore serve to generate the ROS that lead to Top1 cleavage complexes during apoptosis (Figure 1).

A critical question arising from these studies centers on defining the role of apoptotic Top1 cleavage complexes. Top1

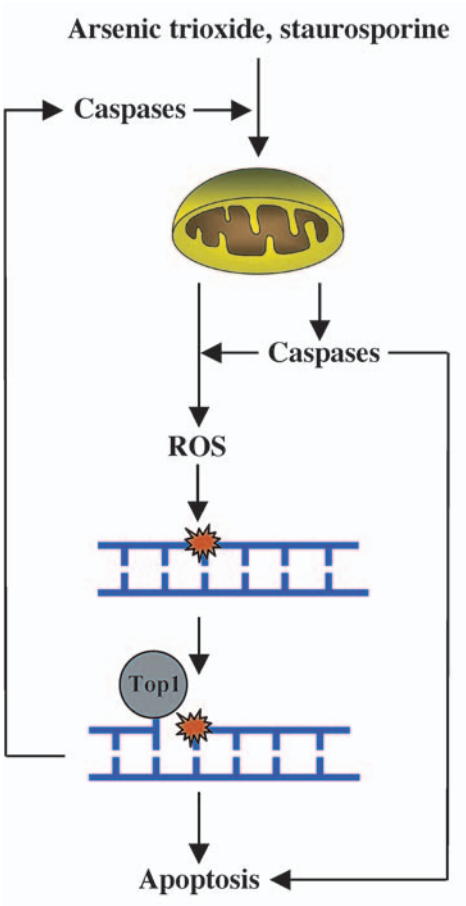

Figure 1. Proposed mechanism for the induction of Top1 cleavage complexes during apoptosis. The non-Top1 inhibitors As_2O_3 and staurosporine induce the generation of reactive oxygen species (ROS) from mitochondria. ROS produce oxidative DNA lesions, which in turn generate Top1 cleavage complexes. These cleavage complexes might participate in apoptosis by degrading chromatin and generating DNA strand breaks. The Top1 cleavage complexes could also engage the apoptotic machinery *in trans* by activating caspases.

downregulation by small interfering RNA (siRNA) reduces DNA fragmentation induced by As_2O_3 and staurosporine. Also, mouse P388/CPT45 cells lacking Top1 show a reduction in apoptotic DNA fragmentation when exposed to As_2O_3 or staurosporine as compared with P388 cells expressing Top1. Thus, Top1 cleavage complexes are likely to contribute to apoptotic DNA fragmentation.

Our findings raise the possibility that Top1, which is abundant and essential in mammalian cells, could participate in apoptosis by directly cleaving chromatin. Like apoptotic endonucleases, Top1 is, however, nonessential since its silencing reduces but does not abrogate apoptotic DNA fragmentation. Top1 cleavage complexes could also engage the apoptotic machinery *in trans,* as trapping of Top1 by camptothecins is among the most efficient inducers of apoptosis (Figure 1) (http://discover.nci.nih.gov/pommier/pommier.htm). Thus, apoptotic Top1 cleavage complexes could serve to amplify the apoptotic process initiated by chemotherapeutic drugs and possibly physiological ligands, including Fas-L (CD95-L, Apo-1L) and TRAIL (Apo-2L).

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■ VIROLOGY

Random or Targeted? Retroviral Integration Target Site Selection for HIV and Murine Leukemia Virus

Wu X, Li Y, Crise B, and Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. *Science* 300: 1749–51, 2003.

When a retrovirus infects a cell, its RNA genome is reverse transcribed into a double-stranded DNA molecule, which is then permanently integrated into the host's DNA. This property makes a retrovirus a good tool for gene delivery, such as a vector for gene therapy. However, the integration of the retrovirus can also lead to insertional mutagenesis.

Studies involving gene therapy for X-linked severe combined immunity deficiencies (X-SCID) have highlighted both the promise and some of the risks involved with the use of retroviruses. In one study, nine of 11 children were successfully treated for X-SCID using a retrovirus carrying a good copy of the gene mutated in the disease. Two of these children, however, later developed leukemias because of retroviral integrations in the putative oncogene *LMO2* (Hacein-Bey-Abina S et al. *Science* 302: 415–9, 2003). It is difficult to explain the high frequency of integrations into the same gene using a random model of retroviral integration. There has been evidence for decades that retroviral integrations may not be random, but the data were somewhat limited in their power to determine the precise nature of the integration biases.

Several early studies suggest that transcription-active regions are preferred sites for retroviral integration (Vijaya S et al. *J Virol* 60: 683–92, 1986; Rohdewohld H et al. *J Virol* 61: 336–43, 1987; Scherdin U et al. *J Virol* 64 :907–12, 1990). However, another study came to the conclusion that transcription-active regions are avoided by the avian leukosis retrovirus (Weidhaas JB et al. *J Virol* 74: 8382–9, 2000). Most of these early studies had very small sample sizes with only a few selected integration sites or were based on specific DNA regions, and thus, their observations might be biased. The completion of the human genome sequence coupled with sensitive PCR techniques makes it possible to paint global pictures of the *in vivo* target site selection for retroviruses.

Using a high-throughput linker-mediated PCR method, we cloned and sequenced thousands of viral-host junction DNA fragments of HIV-1 and Moloney murine leukemia virus (MLV) integration sites from unselected events. The mapping of these sites to the human genome yielded 903 unique MLV and 379 unique HIV integration sites, revealing interesting patterns for HIV and MLV integrations.

Although the results confirm the earlier notion that most or all of the genome is accessible for retroviral integration, HIV and MLV clearly have strong global preferences (Figure 1). To find out if retroviruses prefer to integrate into genes, we used the *RefSeq* gene set, which is based on curated protein-coding mRNAs. For HIV, 58% of the integration sites landed inside *RefSeq* genes, significantly more than a set of computer-simulated random integrations, which resulted in only 22% inside *RefSeq* genes. For MLV, 34% of the integration sites were inside *RefSeq* genes. Although this is significantly higher than that of random integrations (22%), it is also significantly lower than that of HIV-1 (58%). Further analysis showed that MLV has a strong preference for the region surrounding the transcription start site, whereas HIV has no such preference. Corroborating this was the observation that MLV preferred to integrate near CpG islands, which are commonly associated with the transcription start sites of genes. HIV showed no such preference for CpG islands. Microarray expression analysis showed that targeted genes are more actively transcribed for both HIV and MLV.

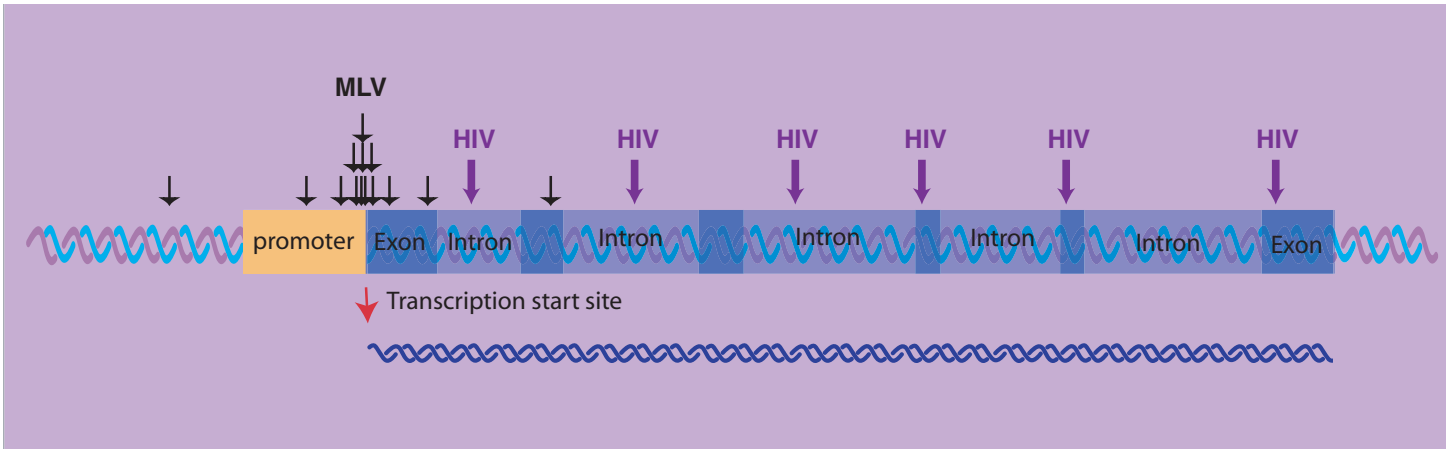

Figure 1. Murine leukemia virus (MLV) and HIV-1 integration preferences. MLV prefers the transcription start sites of genes, whereas HIV-1 prefers anywhere inside of genes.

These results clearly demonstrated that although both MLV and HIV prefer actively transcribed regions, they have marked differences in integration site selection (Figure 1). The strong preference of MLV for the transcription start sites of genes, which are considered the most important regions for gene expression regulation, suggests that gene therapy using an MLV vector may have greater risk than previously thought. It is not surprising anymore that in the two previously mentioned children who developed leukemias, MLV provirus integrated nearby into the 5′ end of the *LMO2* gene and activated the gene, although the cause of leukemia might be more complicated. Our results suggest that integration preferences are specific for each retrovirus, reflecting the idea that different targeting mechanisms might be involved. Thus, it is possible that previous studies that appeared to be contradictory could, in fact, all have been correct in their own way. Their results could simply have reflected the differences in the particular viruses being studied.

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■ IMMUNOLOGY

Molecular Targeting of Graft-versus-Host Disease by Using Proteasome Inhibition

Sun K, Welniak LA, Panoskaltsis-Mortari A, O'Shaughnessy MJ, Liu H, Barao I, Riordan W, Sitcheran R, Wysocki C, Serody JS, Blazar BR, Sayers TJ, and Murphy WJ. Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. *Proc Natl Acad Sci U S A* 101: 8120–5, 2004.

The therapeutic potential of allogeneic bone marrow transplantation (BMT) relies on the graft-versus-tumor (GVT) effect, whereby residual malignant cells of the host are eradicated by the donor T cells. Unfortunately, the clinical use of allogeneic BMT for cancer treatment is seriously hampered by the concomitant occurrence of graft-versus-host disease (GVHD), particularly since the beneficial GVT effects are closely associated with the severity of the GVHD. Any treatment that could reduce the GVHD response, without affecting GVT, could provide for a substantial improvement in allogeneic BMT therapy. GVHD is an immune-mediated disease in which donor T cells recognize and attack the genetically disparate cells of the recipient. It has a complex pathophysiology, ultimately involving multiple organs. Cytokines, particularly inflammatory cytokines produced by T cells and other immune cells, have been shown to be critical for the development of GVHD and play an important role in fueling the entire process.

The transcription factor NF-κB is known to have a pivotal role in cytokine signaling and the generation of cell-mediated immune responses in numerous animal models. Interestingly, the activation of NF-κB can be controlled by the proteasome, so inhibitors of the proteasome can prevent the activation of this transcription factor and subsequently reduce the activities of its downstream target genes. Recently, there has been much interest in clinical application of the proteasome inhibitor, bortezomib (Velcade, formerly PS-341). In previous studies using a murine model of acute

myeloid leukemia, we had observed that bortezomib could sensitize the leukemic cells to apoptotic cell death induced by tumor necrosis factor (TNF)–related, apoptosis-inducing ligand (Apo2L/TRAIL). However, this sensitization of tumor cells to apoptosis did not involve the inhibition of NF-κB (Sayers TJ et al. *Blood* 102: 303–10, 2003). Thus, we hypothesized that bortezomib might be an attractive candidate for administration after allogeneic BMT therapy because of both its ability to sensitize tumor cells to immune-mediated death pathways as well as its potential to limit the severity of GVHD.

In collaboration with Dr. William Murphy of the University of Nevada–Reno, we utilized a model whereby B6 mice received myeloablative radiation followed by intravenous infusion of bone marrow cells from the genetically disparate BALB/c mice. In some instances BALB/c splenocytes were also injected as a source of allogeneic T cells. Initial *in vitro* experiments demonstrated that the inclusion of bortezomib in mixed lymphocyte reactions (MLR) between B6 and BALB/c splenocytes significantly reduced the magnitude of the allogeneic response. Since bortezomib suppressed the MLR reaction *in vitro,* we then assessed whether it could also limit GVHD *in vivo.* After administration of bortezomib on days 0 through 3 following irradiation, BMT, and allogeneic T-cell transfer, survival was significantly extended in this acute model of GVHD. This demonstrated that the early administration of bortezomib was capable of limiting GVHD. A comparison of mice receiving allogeneic T cells in the presence or absence of bortezomib revealed that early engraftment of donor CD4 and CD8 T cells (at 3 and 4 days following transplantation) was significantly reduced by bortezomib. The mechanism underlying this reduced engraftment appeared to be a higher rate of apoptosis of donor T cells in the bortezomib-treated mice. Also, measurements of TNF levels in these mice showed that lower levels of this cytokine were observed in bortezomib-treated mice following BMT.

Although the bortezomib treatment did reduce the effects of GVHD, the crucial question remained as to whether GVT effects were still intact. To assess this, C1498 leukemic cells were injected into the mice 10 days prior to lethal irradiation and allogeneic BMT. In BMT performed in the absence of allogeneic T cells, all mice died of leukemia irrespective of whether they were treated with bortezomib or not. Therefore, any direct inhibitory effects of bortezomib on residual tumor cells were insufficient to provide therapeutic benefit. Tumor-bearing mice receiving irradiation, BMT, and allogeneic T cells but no bortezomib all died of acute GVHD by day 40, whereas bortezomib-treated mice demonstrated a significantly increased survival of up to 75 days. Autopsy of these mice failed to detect any tumor, suggesting that significant anti-tumor effector functions had remained intact. Furthermore, *in vitro* assessment indicated that pretreatment of the leukemia cells with bortezomib rendered them more sensitive to immune-mediated attack.

In summary, the limitation of GVHD most likely involves the inhibition of NF-κB by bortezomib, although further work is necessary to confirm this. For some cancers, the sensitization of the tumor cells by bortezomib to the apoptotic effects of death ligands from the TNF family may provide an additional benefit by enhancing GVT responses. Thus, use of proteasome inhibition with agents such as bortezomib may substantially reduce the severity of GVHD, while having minimal effects on (or even enhancing) the beneficial GVT effects.

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■ CELL BIOLOGY

Bioregulatory Networks That Have Switch-like Behavior: The Hypoxia-response Network

Kohn KW, Riss J, Aprelikova O, Weinstein JN, Pommier Y, and Barrett JC. Properties of switch-like bioregulatory networks studied by simulation of the hypoxia response control system. *Mol Biol Cell* 15: 3042–52, 2004.

Critical cell functions—when to divide, differentiate, die—are controlled by biomolecular networks that are difficult to comprehend, in part because they evolved without the benefit of modular design. Many of their molecular interactions have been discerned. The next big challenge is to understand how they function, a task that demands computer simulation studies. We set our sights on the hypoxia control network because its essential components and connections are known and because its simplest form can be viewed as a single input (the level of molecular oxygen) controlling a single output (the activity of genes controlled by hypoxia response elements [HREs] in their promoters).

Computer-aided simulations are hampered by the complexity of the networks and because the values of the rate constants are unknown. To get useful experimentally testable results, therefore, some plausible assumptions are needed.

First, we assumed that the system of interactions impinging on the key mammalian transcription factor (hypoxia inducible factor [HIF]) has within it a simpler "core" subsystem that already contains the essential response capabilities. The additional interactions then may serve to modulate core system responses according to inputs from other cell control pathways. Not only is simulation of a relatively simple system easier to perform, but more importantly, its results are easier to comprehend. Fortunately, a relatively simple core subsystem of the hypoxia control network is readily identified on the basis of evolutionary conservation. It consists of just four protein species: the two subunits of a transcription factor (HIFα and aryl hydrocarbon receptor nuclear translocator [ARNT]) that turns on HRE-dependent genes; a proline hydroxylase (PHD) that uses molecular oxygen to hydroxylate a proline residue in HIFα; and the von Hippel-Lindau gene product (VHL) that ubiquitinates and causes the degradation of hydroxylated HIFα.

Second, we assumed that the biologically relevant behavior of the system should be robust: It should not be sensitive to the exact values of the rate constant parameters. Thus, the relevant parameters might be found by random search, because evolution might have homed in on suitable parameter sets that are easiest to find. However, we still needed a criterion for "suitable" parameter set. Thus, we invoked a third assumption—that the dependence of the gene activation response on oxygen level should be switch-like. Switch-like behavior is a plausible functional principle that could help to unravel how a system works. Our study demonstrated the power of switch-like behavior in linking theory with experiment and may encourage investigators to carry out quantitative studies to look for this type of behavior.

With these assumptions in place, we searched the parameter space for sets of rate constants that would confer switch-like dependence on oxygen. The first surprise was that the simple core system was indeed capable of switch-like behavior (Figure 1), despite the

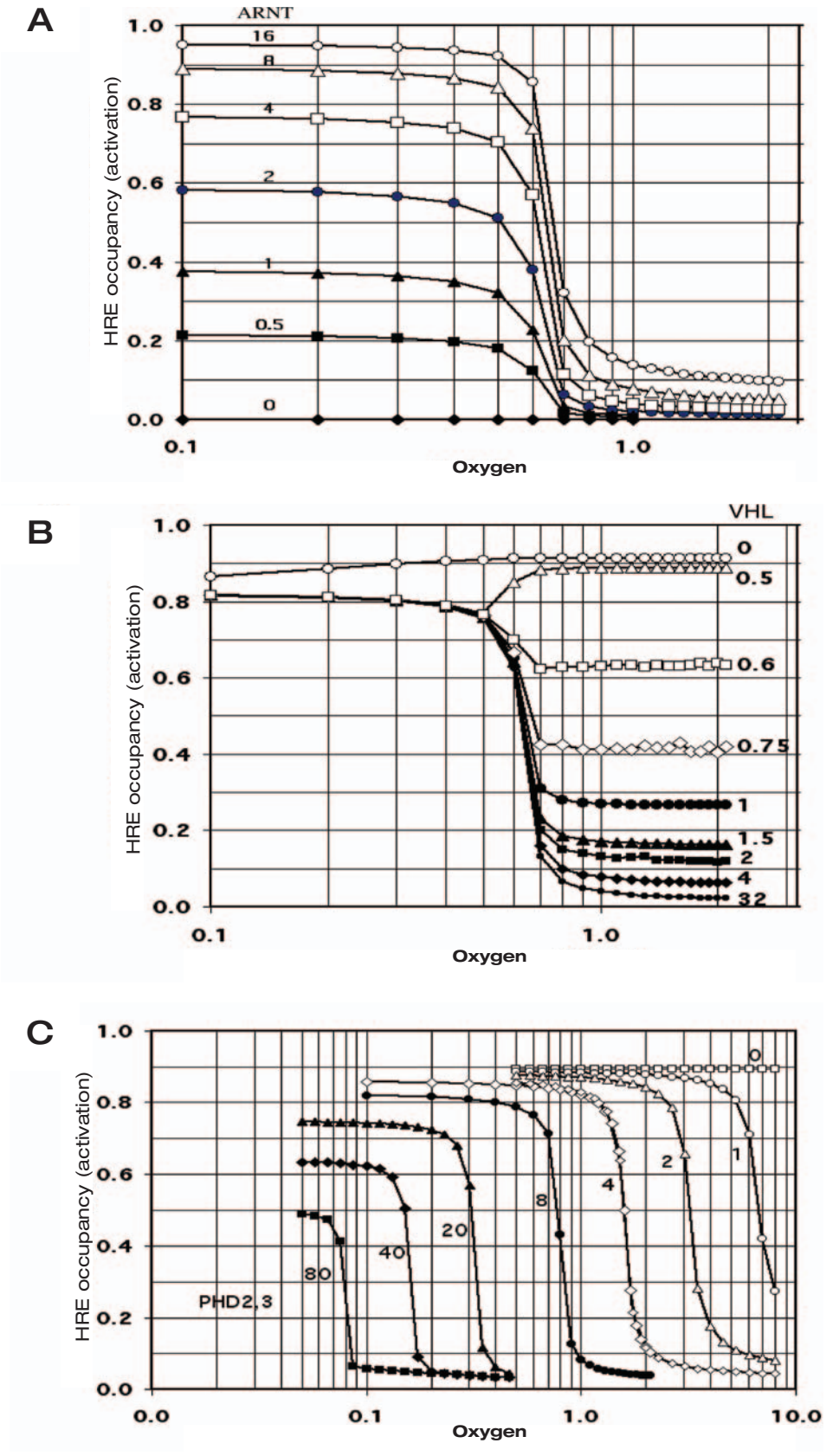

Figure 1. Switch-like behavior in simulations of the "core" hypoxia-response network, showing the predicted dependencies on aryl hydrocarbon receptor nuclear translocator (ARNT) *(panel A),* von Hippel-Lindau protein (VHL) *(panel B),* and proline hydroxylase (PHD) *(panel C).* Activity of hypoxia response element (HRE)–dependent promoters is plotted against oxygen concentration (arbitrary units).

absence of obvious non-linear features, such as feedback or amplification steps. Moreover, there were many sets of rate constants, widely separated in parameter space, that gave switch-like responses, suggesting that such responses are robust.

We found that switch-like response, when it is observed, can provide a powerful link between theory and experiment. For example, simulations showed that oxygen response had a positive dependence on ARNT level under hypoxia, but lack of dependence on ARNT under normoxia, while the oxygen level at the response transition was independent of ARNT level (Figure 1A). The dependence on VHL showed an inverse relationship under normoxia, and lack of dependence under hypoxia, while the oxygen level at the response transition again remained invariant (Figure 1B). On the other hand, the oxygen level at the response transition did depend on the level of PHD (Figure 1C). These theoretical results are amenable to quantitative experimental tests.

To cast light on the mechanism of the switch, we examined the rate constant parameter sets that conferred this behavior. We observed that strong enzyme-substrate binding between PHD and HIFα was required. The mechanism is akin to the Goldbeter-Koshland zero-order ultrasensitivity model. The conjecture of strong enzyme-substrate binding for the PHD, however, remains to be tested.

Our ability to attain these insights attests to the advantage of theoretical studies of the simplest possible subsystem capable of biologically relevant behavior. Our findings show how simulation studies of bioregulatory networks can formulate precise questions for quantitative experiments.

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