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■ MOLECULAR BIOLOGY

Molecular Interaction Maps of Bioregulatory Networks

Aladjem MI, Pasa S, Parodi S, Weinstein JN, Pommier Y, and Kohn KW. Molecular interaction maps—a diagrammatic graphical language for bioregulatory networks. *Sci STKE* 2004: pe8, 2004.

Proper cell growth depends on a complex network of interacting proteins and genes, which regulate crucial activities such as DNA synthesis, gene expression, metabolism, and information processing. Disruptions in the intricate balance between the components of this network may lead to cancer; however, interfering with signals transmitted by bioregulatory networks is an important tool for cancer therapy. In recent years, knowledge about interacting molecules that regulate cell growth has increased exponentially, but our ability to make sense of this detailed information has not. Researchers interested in using modern biology to combat cancer need tools to organize a large collection of facts, including descriptions of bioregulatory molecules, their modifications (for example, phosphorylation), and the complexes they form.

One of the main obstacles to organizing molecular knowledge is the lack of a common language that allows scientists to integrate data in a clear, standardized, and preferably computer-readable format. This article describes a graphical language that encodes molecular information in the form of diagrams, or molecular interaction maps (MIMs) (Figure 1). These MIMs are used to represent and analyze molecular interactions in the same way as circuit diagrams are used to trouble-shoot electronic devices. Investigators usually describe biochemical pathways in cartoon-like diagrams, but these representations of molecular interactions are often incomplete and ambiguous. For example, an arrow between two components could signify an increase in quantity, an increase in activity, or a modification of one molecule by the other. In addition, enzymes in bioregulatory networks are often substrates of other enzymes, and molecules are often subject to modifications that change their binding or enzymatic capabilities. Moreover, regulatory proteins can form multi-molecular complexes, which have different activities, depending on their composition and modifications. Finally, each domain within regulatory molecules may have its own binding, modification, and/or enzymatic functions. Thus, a molecule's activity and interaction capabilities may depend on its modification state, and on the other molecules to which it may be bound. All of these interactions must be taken into account for a full understanding of the system.

In the MIM language, we use a small number of defined unambiguous graphical symbols to portray each type of molecular interaction. Each molecule is represented in a single place in a diagram, and interactions between molecules are specified by arrows or bars at the end of connecting lines. Because modified molecules and multi-molecular complexes may have different properties than the original molecules, the outcome of each interaction (such as a phosphorylated molecule, or a multi-molecular complex) is depicted as a circle, or "node," on an interaction line.

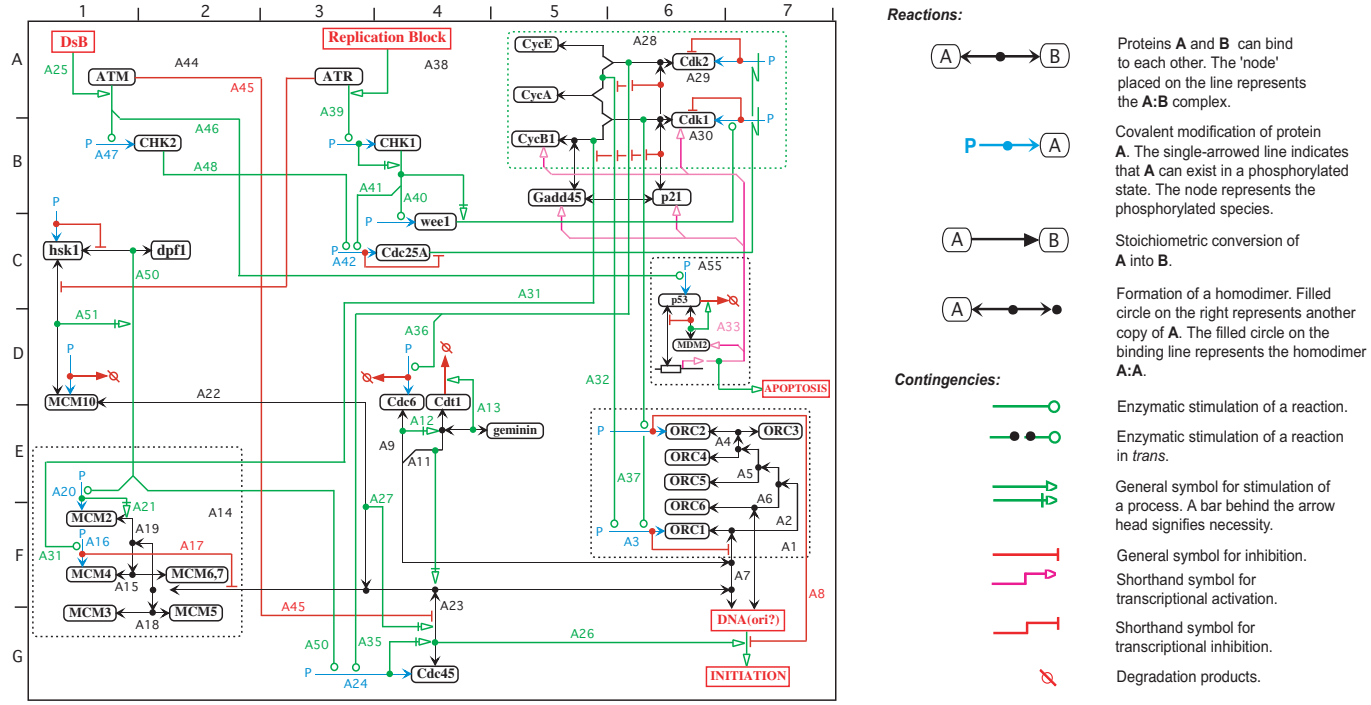

Figure 1. A molecular interaction map portraying the signal transduction network that regulates the onset of DNA replication. Multimolecular complexes or modified forms are depicted by "nodes" placed on the lines. A line may originate either at a named molecular species or at a node, and may terminate at a molecular species, a node, or at another line. Lines that cross do not imply interaction. A detailed description of the symbols of the MIM language is available on the web site, http://discover.nci.nih.gov/mim, in the section "how to read maps." "A" followed by a number represents an annotation (accessible for each MIM at http://discover.nci.nih.gov/mim); ATM, ataxia telangiectasia mutated, a protein kinase that responds to DNA damage; ATR, a relative of ATM, responds to DNA lesions and stalled replication forks; Cdc6, a component of pre-replication complex that recruits Cdt1; Cdc25A, a dual threonine/tyrosine phosphatase; Cdc45, a component of the pre-initiation complex that recruits DNA polymerase; Cdt1, component of pre-replication complex that recruits MCM helicases; CHK1 and 2, serine/threonine kinases that relay DNA damage signals to cell cycle checkpoints; Cdk (1 and 2), a family of cyclin-dependent kinases; cyclins (CycA,E,B1), a family of cell cycle oscillating proteins that bind Cdk proteins; dpf1, the regulatory subunit of hsk1 kinase; DsB, double stranded DNA break; Gadd45, a DNA damage-inducible protein; geminin, an inhibitor of DNA replication that binds Cdt1; hsk1, a kinase that is essential for initiation of DNA replication; MCM(2–7), a helicase that forms a part of the pre-replication complex; MCM10, a protein that binds the hsk1 kinase and the pre-replication complex; MDM2, a protein that binds, regulates, and is regulated by p53; ORC (1–6), an origin recognition complex, bound to chromatin on replication initiation sites; Ori, replication origins, starting sites for DNA replication—there are many origin sites on each chromosome; P (blue), a phosphate group; P21, a regulator of Cdk activity; P53, a tumor suppressor protein often mutated in human cancers; wee1, a protein kinase involved in cell cycle regulation.

These nodes are treated in a way that allows them to form more interactions and extend the network. The symbols and conventions used in the language, as well as examples of MIMs, can be accessed at our web site: http://discover.nci.nih.gov/mim.

The graphical MIM language allows a simultaneous view of many interactions involving any given molecule. It can portray competing interactions, which are common in bioregulatory networks. An interested researcher can trace all the interactions of a given molecule from a single location. Readers can look up a molecule in a glossary or in the electronic (eMIM) diagrams; a mouse-click on the molecule name opens links to more information, including PubMed, CGAP, GeneCards, and Matchminer. Each interaction is labeled with a link to an annotated description, which includes links to cited references. The interested researcher can read the annotations to gain in-depth information on each molecular interaction, or browse the various maps to become acquainted with the general concept of how cells regulate a particular metabolic process.

The full article (referenced above) features four MIMs that describe the molecular interactions that lead to the onset of DNA replication. An electronic version of these MIMs can be found at http://discover.nci.nih.gov/mim/html/index.html. A complete map of all interactions is provided. Additional maps represent subsets of interactions that occur during specific stages of the cell cycle and in response to cellular stress. More maps describing other aspects of bioregulatory signaling will be posted at the same site.

A major task lies ahead to compile and update maps of the major biological control systems, and to integrate them in a concise manner. We may then discern common patterns of molecular interaction logic that give bioregulatory networks their remarkable flexibility and robustness.

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Identification of Novel Human Monoclonal Antibodies to Viral Envelope Glycoproteins and Cancer-associated Antigens and Improvement of Their Efficacy

Zhang MY, Shu Y, Rudolph D, Prabakaran P, Labrijn AF, Zwick MB, Lal RB, and Dimitrov DS. Improved breadth and potency of an HIV-1-neutralizing human single-chain antibody by random mutagenesis and sequential antigen panning. *J Mol Biol* 335: 209–19, 2004.

Polyclonal antibodies have a century-old history of being effective against some viruses; recently, monoclonal antibodies (mAbs) have also shown clinical success (Dimitrov DS. *Nat Rev Microbiol* 2: 109–22, 2004). Although still the only mAb against a viral disease approved by the U.S. Food and Drug Administration (FDA), the humanized mAb palivizumab (Synagis) has been widely used for prevention of respiratory syncytial virus infections in neonates and immune-compromised individuals. Several unmodified mAbs and mAbs armed with toxins or radionuclides have received approval by the FDA for treatment of cancer; more than 400 other mAbs are in clinical trials (Waldmann TA. *Nat Med* 9: 269–77, 2003).

A fundamental problem in the development of effective therapeutic agents against viruses and cancer cells, including therapeutic antibodies, is the cells' and viruses' heterogeneity and mutability. Another problem is that antibodies that cross-react with a broad range of mutants are typically of low binding affinity: One possible solution to this is to identify highly conserved viral structures that are critical for virus entry into cells and that can serve as epitopes—the actual antigenic determinants of antigens to which antibodies bind.

To identify such conserved epitopes, we used complexes of an HIV-1 envelope glycoprotein with CD4 and coreceptor molecules for the screening of human phage libraries (Moulard M et al. *Proc Natl Acad Sci U S A* 99: 6913–8, 2002). In collaboration with Dennis Burton, PhD, of the Scripps Research Institute, a human monoclonal antibody (hmAb) Fab, X5, was identified that exhibited potent and broad neutralizing activity comparable to that of the best characterized potent broadly HIV-1–neutralizing hmAb IgG1 b12. Unlike b12, however, X5, exhibited relatively uniform neutralizing activity when tested on more than 50 primary isolates. Our collaborator Xinhua Ji, PhD, (CCR, NCI-Frederick) and his associates solved the crystal structure of X5 and found a long protruding flexible CDR3 of the antibody's heavy chain that appears to be critical for the antibody's high binding affinity; the amino acid residues forming the epitope to which X5 binds, as identified by alanine scanning mutagenesis, were highly conserved, which offers a possible explanation for X5's broad neutralizing activity (Darbha R et al. *Biochemistry* 43: 1410–7, 2004).

To further improve the binding affinity of X5 without losing its cross-reactivity, and to enhance the selection of novel broadly reactive hmAbs, we developed an approach based on sequentially changing antigens during antibody selection—termed sequential antigen panning (SAP) (Zhang MY et al. *J Immunol Methods* 283: 17–25, 2003). Several antigens representing different viral isolates were sequentially changed during the panning procedure leading to the selection of antibodies against epitopes shared among these antigens. This approach was used for the selection of several broadly cross-reactive hmAbs, including m16 (Zhang MY et al. *Antiviral Res* 61: 161–4, 2004) and m14 (Zhang MY et al. *J Virol* 78: 9233–42, 2004). To further improve the binding affinity of X5, we generated a mutant X5 library and used the SAP approach for the selection of high-affinity antibodies that bound to all antigens used for the panning and screening (Zhang MY et al. *J Mol Biol* 335: 209–19, 2004).

A single-chain hmAb selected by this procedure, m9, was extensively tested for its binding and inhibitory activity (Zhang MY et al. *J Mol Biol* 335: 209–19, 2004, and unpublished data). Its binding affinity was on average 2- to 4-fold higher with a 50-percent inhibitory concentration (IC_{50}) 2- to 10-fold lower than that of X5. Importantly, more primary HIV-1 isolates from different subtypes were neutralized by m9 than by X5. Thus, both the potency and breadth of neutralization were improved. m9 neutralized more than 50 primary isolates from different HIV-1 genetic subtypes including clade C, which is the dominant subtype around the world, and clade B, which is dominant in the United States. To date, only several other potent broadly HIV-1–neutralizing hmAbs (b12, 2G12, 447-52D, X5, 2F5, and 4E10/Z13) are known of the large number of antibodies tested, and it appears that m9 exhibits exceptional potency and breadth of neutralization. (For example, for a panel of 17 clade C primary isolates, it was superior to any

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other antibody tested.) NCI has filed three patent applications. Two licenses of these applications were executed with Tanox Pharmaceuticals, Inc. for m9 and several other hmAbs selected by SAP (m6, m12, m14, m16, and m18), and one license with Virosys Pharmaceuticals, Inc. for X5. More than 30 investigators from the United States, Europe, and Australia have requested and received them.

The identification and characterization of novel broadly neutralizing hmAbs and their epitopes could also help in the development of vaccine immunogens that could elicit the same antibodies *in vivo*. This approach, termed retrovaccinology (Burton DR. *Nat Rev Immunol* 2: 706–13, 2002), contrasts with standard approaches based on evaluation of the antigen first. We found that some mutations in the HIV-1 envelope glycoproteins significantly increased binding of the antibodies we identified; these mutated glycoproteins may have potential as vaccine immunogens. A large amount of work based on a variety of antigens failed to elicit *in vivo* any of the few known potent broadly HIV-1–neutralizing antibodies; perhaps such mutated envelope glycoproteins could help solve this problem that is of major importance for vaccine development.

A number of similarities exist in the strategies used by cancer cells and viruses causing chronic diseases (e.g., HIV) regarding the evasion of immune responses as well as in the mechanisms of the ligand-receptor interactions leading to virus entry and signal transduction across membranes. We are currently developing hmAbs against components of the insulin-like growth factor (IGF) system using approaches similar to those we have developed for anti–HIV-1 hmAbs. We have recently identified two high-affinity hmAbs against IGF-II and, in collaboration with J. Carl Barrett, PhD, (CCR, NCI) and his associates, are characterizing them as potential candidate cancer therapeutics.

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■ TRANSLATIONAL RESEARCH

Dual Role of Transforming Growth Factor-β Signaling in Breast Cancer

Tian F, DaCosta Byfield S, Parks WT, Yoo S, Felici A, Tang B, Piek E, Wakefield LM, and Roberts AB. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 63: 8284–92, 2003.

The compound transforming growth factor-β (TGF-β) received its name due to its ability to transform normal fibroblasts by inducing their anchorage-independent growth. This *in vitro* "transforming" or presumed pro-oncogenic activity of TGF-β is now known to predominate only in late stages of carcinogenesis, manifesting itself as a pro-metastatic activity and involving (1) reduction of cell adhesion (sometimes associated with epithelial-to-mesenchymal transition [EMT]), (2) increased cell mobility, and (3) increased production of matrix-degrading proteins such as the metalloproteinases, typically associated with invasive activity. Paradoxically, TGF-β also has potent tumor suppressor activity, based in part on its ability to inhibit the growth of most epithelial and lymphoid cells, which form the basis of most human cancers. This activity is now thought to predominate in the earlier stages of cancer progression, stages during which the cells remain sensitive to the growth inhibitory effects of TGF-β, having not yet reached the point of unchecked proliferation.

The unanswered question is how TGF-β might switch from a tumor suppressor to a pro-metastatic agent. An important, known fact is that the expression levels of the TGF-β transmembrane receptors are reduced as cells progress from early to late stages of carcinogenesis. Consistent with this reduction in receptor levels, end points such as growth inhibition, which are known to require robust signaling over a period of at least 8 to 10 hours, cannot be sustained. In addition to changes in signaling strength and duration, it is also important to know the extent to which the signaling context is changed as cells progress from a premalignant state to one in which they are fully invasive and metastatic.

TGF-β acts via unique transmembrane receptor serine/threonine kinases. Signals from these receptors are transduced primarily via a family of latent transcription factors called Smad proteins. In the case of TGF-β, Smad2 and Smad3 are direct substrates of the type I receptor kinase, being activated by phosphorylation on a C-terminal serine motif. Once activated, they partner with the common mediator Smad4 and are translocated to the nucleus where they regulate transcription of target genes in collaboration with a wide variety of sequence-specific transcription factors. Not only may the balance between Smad2 and Smad3 signaling change during the course of carcinogenic transformation of cells, but the balance between Smad signaling and that of other pathways activated by TGF-β, such as the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) signal transduction pathways, may also change, such that a shift in pathway utilization may occur as TGF-β changes from tumor suppressor to pro-metastatic activity (Figure 1).

To address these issues, we have used a set of cells derived from Ras-transformants

of human MCF10A cells. Although all these cells have a common origin, they represent defined stages in cancer progression—from premalignant to well differentiated to highly invasive and metastatic. We asked whether the Smad signaling pathway can mediate both tumor suppressor and pro-metastatic effects in these cells. To investigate this, we used stable infection with retroviral constructs to either (1) enhance the expression of Smad3 to mimic a change in the balance of Smad2/3 signaling in cells in which Smad2 is mutated and functionally inactive or (2) block signaling through both Smad2 and Smad3 to mimic epigenetic suppression of this pathway seen in many cancer cells. The surprising outcome was that overexpression of Smad3 suppressed tumorigenesis, as determined by subcutaneous injection of well-differentiated MCF10CA1h cells into nude mice, yet this identical manipulation in the metastatic MCF10CA1a cells promoted lung metastases following tail-vein injection. Inhibition of the Smad pathway had opposite effects, enhancing tumorigenesis of the MCF10CA1h cells and suppressing metastasis of the MCF10CA1a cells. Repression of Smad signaling in MCF10CA1a cells also blocked effects of TGF-β on relocalization of cell-surface E-cadherin, commonly associated with EMT. Together, these data suggest that the *same* pathway can mediate both the tumor suppressor and pro-metastatic activities of TGF-β. This pathway may operate in conjunction with a shift in balance of Smad signaling and other pathways or with an altered cellular context, as could be effected by a change in the utilization or expression of transcriptional cofactors.

What are the implications of these findings for new therapeutic approaches to cancer? Many studies, both by our colleague and collaborator Lalage Wakefield, DPhil (Tang B et al. *J Clin Invest* 112: 1116–24, 2003) using this same set of MCF10A-derived cells, and by others using models based on spontaneous tumorigenesis in Neu oncogene-expressing mice (Siegel PM et al. *Proc Natl Acad Sci U S A* 100: 8430–5, 2003), have shown that interference with TGF-β signaling at the level of the ligand or the receptors has

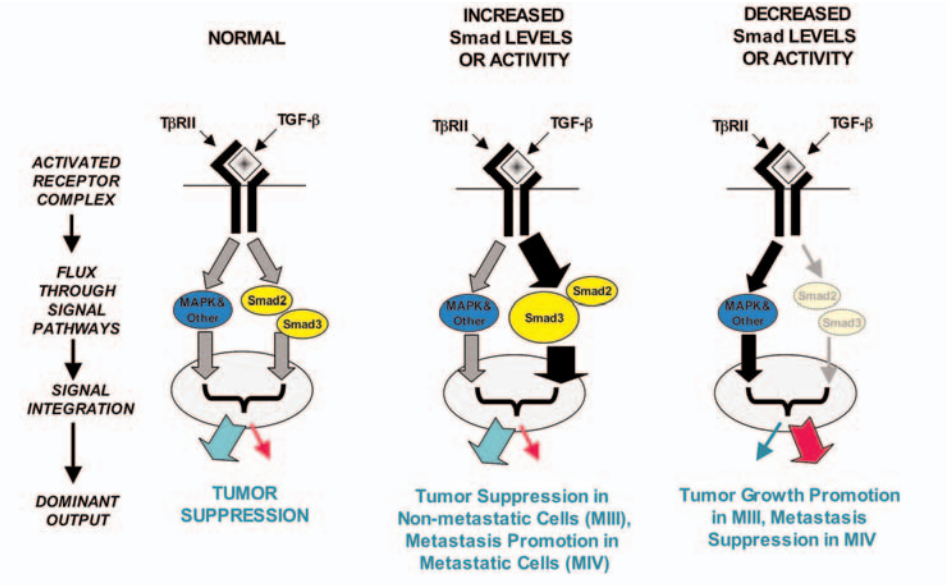

Figure 1. Balance of normal transforming growth factor-β (TGF-β) signaling flux is modulated by changing Smad2/3 activity, which shows the opposing effects in non-metastatic breast cancer MIII cells compared with metastatic MIV cells. The same signaling pathway mediates *both* tumor suppressor *and* pro-metastatic signals depending on the cellular context. TβRII, transforming growth factor-β receptor II; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

the same dual effects on tumor suppression and pro-metastatic activity that we have observed by direct manipulation of the Smad signaling pathway. We interpret this to mean that effects of the Smad pathway, possibly even of Smad3 specifically, are dominant in carcinogenesis. Moreover, given the intense interest at present by biotech companies in developing both anti-ligand approaches and small molecule inhibitors of the TGF-β type I receptor kinase, there is promise that these approaches, used in conjunction with appropriate molecular diagnostics and likely in combination with other kinase-directed approaches or cytotoxic therapies may be effective in preventing or suppressing metastatic disease.

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Elevated Levels of Thymidylate Synthase Linked to Cancer Etiology

Rahman L, Voeller D, Rahman M, Lipkowitz S, Allegra C, Barrett JC, Kaye FJ, and Zajac-Kaye M. Thymidylate synthase as an oncogene: a novel role for an essential DNA synthesis enzyme. *Cancer Cell* 5: 341–51, 2004.

The biochemical properties of thymidylate synthase (TS), an enzyme that plays a critical role in DNA synthesis, have been extensively studied for more than 40 years, but much less is known about a biological role for the elevated levels of TS observed in many human tumors. Our recent work shows that an ectopic overexpression of TS may result in deregulated cell growth and cancer.

TS plays a central role in the biosynthesis of thymidylate, 2′-deoxythymidine-5′-monophosphate (dTMP), an essential precursor for DNA synthesis (Figure 1). Because TS activity generates the sole intracellular *de novo* source of dTMP, TS is essential for cellular proliferation and growth. Clinical studies have shown that TS levels are elevated in cervical, breast, kidney, bladder, lung, and gastrointestinal tumor tissues as compared with their normal counterparts and that high TS levels have been associated with a worse clinical outcome in these cancers. Elevated steady-state levels of TS could therefore arise as a secondary effect of tumor formation and/or could have a direct or indirect role in the tumorigenesis pathway.

To test whether ectopic overexpression of TS results in cellular transformation, we transfected mouse NIH3T3 cells with an expression plasmid encoding human TS and observed that elevated levels of TS caused immortalized NIH3T3 cells to acquire phenotypic characteristics of cancer cells as manifested by foci formation, anchorage independent growth, and tumor formation in nude mice. In addition, a direct association of TS with a transformed phenotype was demonstrated by using both a tetracycline-inducible TS promoter and small interfering RNA (siRNA) directed against TS. We also showed that overexpression of human TS in E1A immortalized rat kidney cells (RK3E) resulted in transformation of these cells, demonstrating that elevated levels of TS can induce transformation in two distinct mammalian cell lines. The acquisition of the transformed phenotype was dependent on the catalytic activity of TS, considering that similarly elevated levels of functionally inactive TS mutants did not have the same transforming effect. These observations suggest that there may be a link between TS-mediated DNA synthesis and the transforming ability of TS.

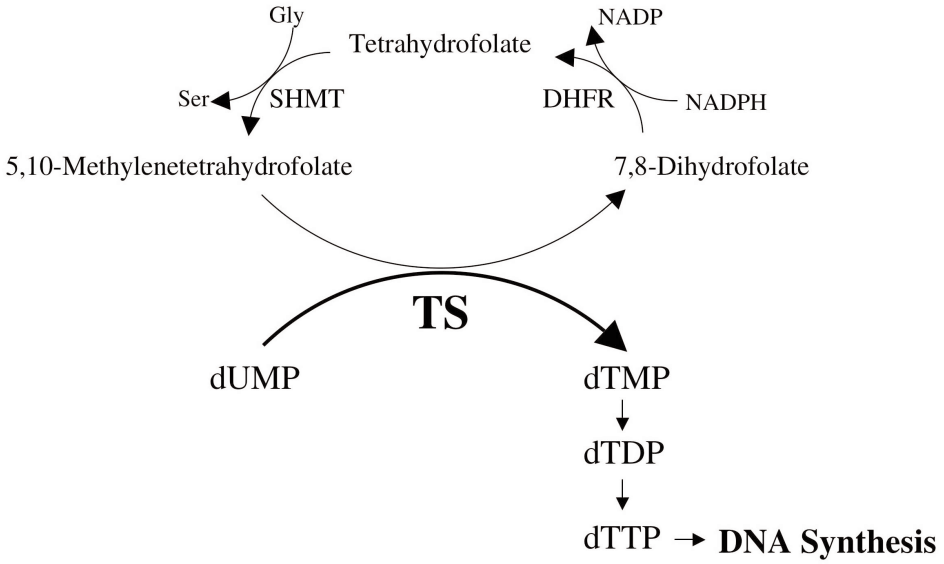

Figure 1. Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine 5-monophosphate (dUMP) by transfer of a methylene group from a cofactor (5,10-methylenetetrahydrofolate) to generate thymidylate (2′-deoxythymidine-5′-monophoshate [dTMP]). dTMP is further phosphorylated to the triphosphate state (dTTP), which is a direct precursor for DNA synthesis. Continuous synthesis of dTMP requires regeneration of 5,10-methylenetetrahydrofolate from 7,8-dihydrofolate. This occurs by two steps catalyzed by the enzymes dihydrofolate reductase (DHFR) and serine hydroxymethyltransferase (SHMT).

...an ectopic overexpression of [thymidylate synthase] may result in deregulated cell growth and cancer.

Because overexpression of oncogenes has been shown to induce programmed cell death, we asked whether TS overexpression could also promote programmed cell death upon serum deprivation. We observed that TS-overexpressing transformed cells showed increased susceptibility to apoptotic cell death following removal of serum from their growth medium, as measured by poly(ADP-ribose) polymerase (PARP) cleavage and by TUNEL assay. Elevated levels of TS may lead to accumulation of genetic alteration due to deoxyribonucleoside triphosphate (dNTP) imbalance that can result in enhanced susceptibility to apoptotic cell death upon serum removal or a transformed phenotype. Although the mechanism of how these elevated levels of TS might participate in apoptotic cell death and in the neoplastic transformation process remains to be defined, our work suggests that elevated levels of TS may play an important role in tumor development.

Since ectopic expression of E2F-1 has been shown to stimulate the expression of TS, we hypothesized that TS may act as a downstream effector of E2F-1, and thus it may at least partially mediate E2F-1 oncogenic activity. The finding that high levels of TS were associated with the transformation of murine cells toward a malignant phenotype supports the continued development of new reagents to interfere with the function and the elevated steady-state levels of TS. However, the available pharmaceutical drugs that are used clinically as substrate analogs to inactivate the catalytic activity of TS are also known to increase TS levels. Thus, development of drugs that target E2F-1 transcriptional activity combined with agents that block the catalytic function of TS would provide new avenues for the design of future strategies for cancer prevention and treatment.

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CANCER AND CELL BIOLOGY

The Glue Signals Too

Qian X, Karpova T, Sheppard AM, McNally J, and Lowy DR. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J* 23: 1739–84, 2004.

E-cadherin is a tumor suppressor whose expression is frequently silenced in human cancers (Hajra KM et al. *Genes Chromosomes Cancer* 34: 255–68, 2002). In normal cells, E-cadherin maintains the integrity of virtually all epithelial tissues through its ability to form cell-cell adhesions via interactions between E-cadherin molecules on the surface of adjacent cells. E-cadherin may also regulate signaling, but no adhesion-dependent signaling activity had previously been clearly identified: We have now made the unexpected observation that E-cadherin can negatively regulate the activation of several classes of receptor tyrosine kinases (RTK), including epidermal growth factor receptor (EGFR)/neu, insulin-like growth factor-1 receptor (IGF-1R), and hepatocyte growth factor (HGF) receptor (c-Met). This adhesion-dependent activity represents a new function for E-cadherin. As the activities of many RTKs have been implicated in the pathogenesis of a wide range of cancers, loss of this form of negative RTK regulation may account, at least in part, for the frequent selection of silenced E-cadherin expression in cancer.

The initial observations were made with endogenous E-cadherin and RTKs in the epithelial Madin-Darby canine kidney (MDCK) cell line, which is widely used to explore epithelial cell physiology. When the cells were confluent, the density-dependent inhibition of their growth was correlated with a strong inhibition of ligand-dependent RTK activation. The RTK inhibition was found to have resulted from a severe reduction in the binding affinity of RTK ligands, as determined by Scatchard analysis, without a concomitant reduction in the number of binding sites or a reduction in the surface accessibility of the RTKs. The inhibition of ligand binding and of RTK activation was E-cadherin dependent, as the activity was specifically abrogated by a neutralizing E-cadherin antibody. Consistent with the inhibition being attributable primarily to impaired ligand-receptor interaction, E-cadherin did not regulate a mutationally activated neu oncoprotein whose constitutive activity is ligand independent. The negative regulation did not extend to G-protein–coupled receptors (GPCR), since the ligand-dependent activation of two classes of GPCR—lysophosphatidic acid (LPA) and muscarinic receptors—was not regulated by E-cadherin.

The findings in MDCK cells were extended to two human tumor cell lines—a melanoma line and a breast cancer line—that did not express endogenous E-cadherin (Figure 1). When E-cadherin was transfected into these lines, it inhibited their anchorage-independent growth and conferred ligand-dependent RTK regulation similar to that observed in confluent MDCK cells, whereas ligand-dependent GPCR activation was not regulated by the E-cadherin.

RTK regulation by E-cadherin was associated with the colocalization of both classes of protein to the basolateral region of polarized cells. Furthermore, E-cadherin was found to form a complex with both EGFR and IGF-1R in MDCK cells, as determined by immunoprecipitation and Western blotting. The cytoplasmic portion of E-cadherin possesses two well-characterized sites for noncovalent binding to heterologous proteins: a C-terminal domain that binds beta-catenin and a more proximal domain that binds p120-catenin. When E-cadherin mutants deficient for binding either beta-catenin or p120-catenin were transfected into human embryo kidney 293 (HEK293) cells that stably express EGFR, E-cadherin-EGFR complexes were formed with the mutants as efficiently as with wild-type E-cadherin, and both mutants retained some adhesion activity and the ability to negatively regulate RTKs. By contrast, an E-cadherin mutant lacking most of the extracellular domain did not form complexes with EGFR, did not have adhesive activity, and did not regulate RTKs. Complex formation between EGFR and E-cadherin was specific, in that

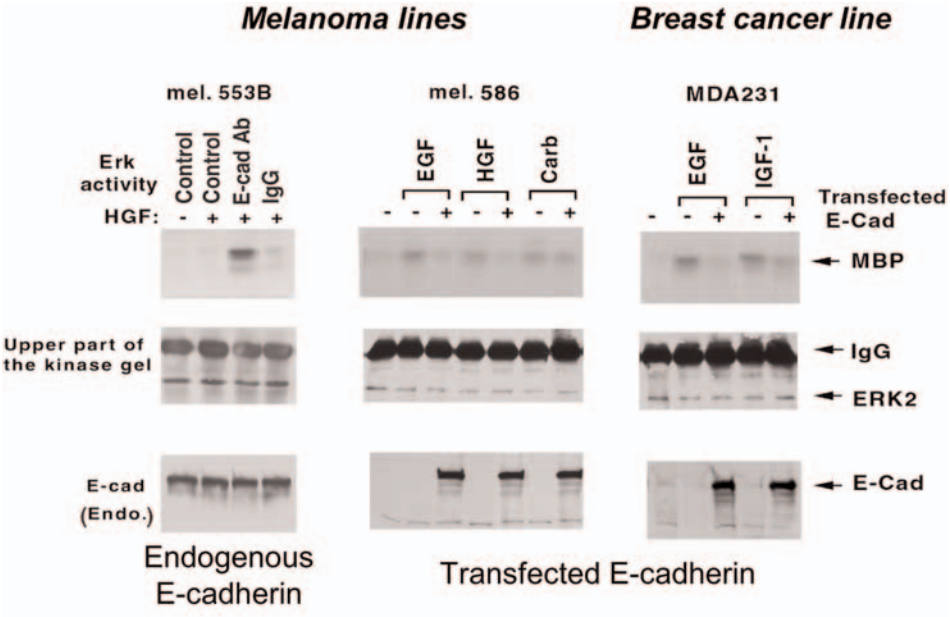

Figure 1. E-cadherin in human tumor cell lines regulates RTK activation. One cell line was a human melanoma line (mel. 553B) expressing endogenous E-cadherin (left panels). In addition, there were two lines with downregulated endogenous E-cadherin that had been transfected with E-cadherin: a human melanoma line (mel. 586, middle panels) and a human breast cancer line (MDA231, right panels). The lines were stimulated for 5 minutes with the indicated growth factor, with the mel. 553B line having first been treated with a neutralizing E-cadherin antibody (E-cad Ab) or control immunoglobulin G (IgG). Cells were analyzed for extracellular signal-regulated kinase (ERK) activity (myelin basic protein [MBP], upper panels) and for ERK protein loading (middle panels, horizontally). Extracts were also immunoblotted with an anti–E-cadherin antibody (bottom panels). IGF-1, insulin-like growth factor; EGF, epidermal growth factor; carb, carbachol; HGF, hepatocyte growth factor.

complexes were not detected between EGFR and N-cadherin, or between E-cadherin and a GPCR muscarinic receptor. Together, these findings indicate that the interaction between E-cadherin and EGFR is specific and requires the extracellular domain of E-cadherin. The interaction between E-cadherin and EGFR is not, however, mediated by the interaction between E-cadherin and its two well-recognized binding partners, beta-catenin and p120-catenin. Furthermore, it is independent of the sites in E-cadherin to which these proteins bind. We can therefore infer that the interaction between E-cadherin and EGFR involves a site in E-cadherin not previously identified as mediating interactions with heterologous proteins.

We conclude that negative regulation of RTKs by E-cadherin is a physiologic adhesion-dependent activity that results from a reduction in the efficiency of RTK ligand binding. The activity is abrogated in tumors when E-cadherin is downregulated. It can be restored when E-cadherin is reexpressed. The pleiotropic inhibitory effects of E-cadherin imply that restoration of its expression in cancers in which it has been silenced may represent a potent means for reversing the malignant phenotype.

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■ VIROLOGY

Nonrandom HIV-1 Infection and Double Infection

Dang Q, Chen J, Unutmaz D, Coffin JM, Pathak VK, Powell D, KewalRamani VN, Maldarelli F, and Hu WS. Nonrandom HIV-1 infection and double infection via direct and cell-mediated pathways. *Proc Natl Acad Sci U S A* 101: 632–7, 2004.

One of the major difficulties in treating HIV-1 infection and generating an effective vaccine is the high rate of genetic variation in the viral population. This variation promotes the generation of viral strains that become resistant to drugs or that escape the host immune response. Three inherent features of HIV-1 replication contribute to the high variation: recombination, mutations, and the number of virus replication cycles in the host.

HIV-1 has one of the highest recombination rates among viruses; for example, markers separated by 1.3 kb can segregate as unlinked sequences during one round of viral replication. However, this powerful mechanism to generate genetic variation has a prerequisite: the formation of virions containing two RNA molecules that are different (heterozygotes). HIV-1 packages two copies of viral RNA into one virion, and frequent recombination occurs during reverse transcription between the two copackaged RNAs. Hence, recombination can only be observed when the two copackaged RNA molecules are genetically different. Heterozygous virions are exclusively generated from cells that are infected by more than one retrovirus. Therefore, the frequency of cells infected with more than one virus (double infection) directly affects the observed recombination events.

Currently, very little is known about the kinetics of double infection. If infection

events are random, then the frequency of double infection can be calculated based on the frequencies of infection. For example, if two populations of viruses are present and each has a multiplicity of infection (moi) of 0.1 infectious units per cell, then an estimated 1% of the cells would be infected by both viruses. However, the assumption that all infection events are random also implies that all target cells are equally susceptible to infection. If all cells were not equally susceptible to HIV-1 infection, then double infection would not be random.

We directly probed the dynamics and nature of double infection using an assay that allowed only a single round of virus replication. Viruses generated from two HIV-1 vectors containing different markers were mixed together and used to infect target cells. The numbers of cells expressing none, one, or both markers were determined and used to calculate whether double infection occurred at frequencies expected from random infection events. We found that double infection occurred significantly more frequently than predicted from random distribution in all experiments using a target T-cell line. In addition, we observed increased rates of double infection in primary activated $CD4^+$ T cells in multiple experiments, each using cells collected from a different donor. The level of increased double infection varied among primary T cells isolated from different donors and could reach 10-fold higher than the expected double infection rate based on random distribution. We have also examined double infection using a large range of moi, and at all moi tested, double infection occurred at a higher frequency than expected from random events. These experiments indicated that HIV-1 infection is nonrandom, thereby causing the observed nonrandom double infection. We hypothesized that variation in susceptibility to virus infection exists in the target-cell population and causes the nonrandom double infection. We have also performed mathematical simulations to illustrate that variation in the infectability of the target cells could cause nonrandom double infection as we observed.

In addition to infecting cells directly, HIV-1 can be transmitted via a cell-mediated pathway; for example, dendritic cells (DCs) can capture HIV-1 particles and later transmit the viruses to target cells. We also examined the nature of cell-mediated HIV-1 double infection by using primary human DCs to capture and transmit HIV-1. We observed increased HIV-1 double infection by the cell-mediated pathway; the level of increased double infection also varied among donors and could be up to 14-fold higher than anticipated from random distribution. Image analyses from our laboratory and others revealed that human DCs can have multiple virion-containing cellular compartments; additionally, multiple virions can be present between the contact points of a DC and a T cell during cell-cell interaction. Our double infection data, together with the image analyses, led us to hypothesize that multiple HIV-1 virions are transmitted from a DC to a T cell, thereby resulting in the increased double infection observed in our analyses.

Together, our results indicate that HIV-1 double infection occurs more frequently than expected from random events in both direct and cell-mediated HIV-1 infections. To our knowledge, this is the first direct evidence of nonrandom infection and double infection in HIV-1 and in retroviruses. Frequent double HIV-1 infections in patients would allow the generation of recombinant viruses that could then affect their pathogenesis and evolution. Increased double infection also implies that recombinant HIV-1 is generated in persons with the infection at rates higher than previously anticipated. Interestingly, analyses of spleen samples from two patients revealed that 75% to 80% of HIV-1–infected cells harbored more than one provirus (Jung A et al. *Nature* 418: 144, 2002). This observation agrees with our study and lends further support to the hypothesis that double infection occurs far more frequently than it would at random in infected individuals. These results have significant implications for understanding the *in vivo* dynamics of HIV-1 infection in patients.

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■ ADMINISTRATIVE LINKS

NIH Password Policy Change
Effective January 1, 2005, NIH implemented a new computer password policy. When your current password expires, you will be required to select a new password that is at least seven characters in length and uses a combination of at least three of the following: uppercase letters, lowercase letters, numeric characters, and special characters. You will also be required to change your password every 180 days.

NCI Express Services
NCI staff located on the Bethesda campus may obtain minor facility services via the NCI Express Facility Services online request form at http://camp.nci.nih.gov/admin/osfm/express.html. Help is available for hanging clocks, pictures, etc.; carpet repair, replacement, and cleaning; painting and wall repair; moving workstations and files; custom millwork (shelves and cabinets); and other tasks.

Mentoring: An Important Part of CCR's Mission

A critical aspect of CCR's mission is training future generations of scientific leaders. Achieving independence in science requires strong focus, commitment, and mentorship. Mentoring is important in helping investigators take full advantage of the training opportunities at CCR, while contributing to the advancement of cancer research. Conscientious mentors proactively encourage trainees to seek the resources, educational opportunities, and experiences needed for a successful career. Mentoring extends well beyond providing laboratory training and career advice—the ultimate goal of mentoring is to facilitate the young investigators' transition to recognized independence.

The approach for facilitating this transition depends greatly on the trainee. The proportion of mentoring support and direction changes as the trainee's level of competence, self-confidence, and expertise grows over time. Successful mentors recognize the strengths and weaknesses of the fellow and continually challenge the individual to advance his or her knowledge base and independence.

An ideal mentoring relationship will progress over time from one in which a greater degree of guidance and discussion is needed and desired by both parties to one in which the trainee is largely functioning as a peer. Whereas trainees are likely to arrive with strong competence in their research area, expanding those skills as well as acquiring new ones will be their focus for the next several years. The mentor can reward the individual strengths of the fellow by giving more independence in those areas while simultaneously recognizing those that require improvement and providing support, guidance, and nurturing to ensure they flourish. Trainees will come to recognize that they can make significant accomplishments through their own explorations, research, and discussions with experts. By embracing opportunities for growth and accepting new challenges, trainees will develop their own patterns of behavior and ways of operating, which will eventually reflect their readiness for independence.

Based on the above mentoring approach, one can discern several principles for success:

Motivation—Successful mentoring requires a commitment to the trainee's career; if mentor or trainee encounters difficulties, the individual should consult available resources and seek creative solutions.

Achieving independence in science requires strong focus, commitment, and mentorship.

Communication—Successful mentoring involves continuous discussion of the training goals and career directions, as expectations and goals might change over time. To facilitate this, CCR has developed a training plan form for the trainee to complete annually and use as a basis for discussing research progress with the mentor. The questions in the form have been carefully selected to encourage discussion and evaluation of the trainee's goals, objectives, and needs by both the mentor and the trainee. Trainees should feel comfortable discussing expectations such as level of independence, number of publications, time at the bench, attendance at meetings, and ability to take projects to future academic positions.

Flexibility—In a successful mentoring relationship, both the trainee and mentor are willing to change course and explore new opportunities if a particular research direction is not promising.

Networking—Successful mentors appreciate the skills and enthusiasm trainees bring to science and provide opportunities to enhance the trainees' visibility by sending them to give talks or presentations on their own, introducing them to experts in the field, and encouraging them to seek out subject experts and establish their own scientific relationships.

Risk-taking—It is not immediately obvious how much risk to take as one moves forward on the career path. In a good mentoring relationship, the trainee will learn how to approach risk-taking within his or her project portfolio such that a proper balance is achieved and maintained.

Whereas the primary mentoring relationship is often between the principal investigators and their trainees, a mentor can be anyone with strong scientific knowledge and a commitment to the trainee's career development. In fact, trainees are encouraged to seek secondary mentors to serve as an alternate, complementary source of instruction.

Mentoring skills are part of an investigator's performance evaluation and are among the criteria for the *Outstanding Performance Award*. To promote mentoring, the NCI Director annually presents the *Outstanding Mentor* and *Mentors of Merit* Awards to investigators who exhibit superior mentoring skills. Also, CCR's Office of Training and Education is developing a *Mentoring Handbook* for both CCR mentors and fellows.

In summary, successful mentors know the individual traits of their trainees and strive to meet their evolving needs and demands. Their approach changes gradually with career growth, leading to mutual trust and respect. Mentoring success promotes and maintains high standards in research and prepares the future generation of leaders in cancer research for the challenges that await them.

J. Carl Barrett, PhD
Director

Loss of BRCA2 in Adult Gonads Reveals Its Sexually Dimorphic Role in Gametogenesis

Sharan SK, Pyle A, Coppola V, Babus J, Swaminathan S, Benedict J, Swing D, Martin BK, Tessarollo L, Evans JP, Flaws JA, and Handel MA. BRCA2 deficiency in mice leads to meiotic impairment and infertility. *Development* 131: 131–42, 2004.

Germline mutations in *BRCA2*, the human breast cancer susceptibility gene, are responsible for approximately half of all familial breast cancer cases. BRCA2 acts as a tumor suppressor by maintaining genomic integrity. To date, its role is well established only in the RAD51-mediated double-strand break-repair process (Patel KJ et al. *Mol Cell* 1: 347–57, 1998). It has been proposed that BRCA2 may bind to RAD51 and hold it in an inactive state (Davies AA et al. *Mol Cell* 7: 273–82, 2001). When the DNA is damaged, BRCA2 recruits RAD51 to the site and repair is initiated. Given the essential role of BRCA2 in DNA repair and its expression in multiple tissues, it is puzzling that germ-line mutations in this gene result predominantly in breast and ovarian cancer and not the plethora of other phenotypes that can be imagined. Interestingly, while BRCA2-deficient breast and ovarian epithelial tissues undergo uncontrolled growth and result in tumorigenesis, BRCA2-deficient mouse embryos and embryonic stem cells undergo cell death instead (Sharan SK et al. *Nature* 386: 804–10, 1997). This suggests that the response of the cells in coping with damaged DNA may be tissue specific and could be a key factor in determining their fate. When the DNA is damaged, cells have three choices: continue to proliferate without DNA repair, repair damage before resuming the cell cycle, or undergo cell death. Perhaps cells with stringent cell-cycle checkpoints will select one of the latter two options, while the first path may be undertaken by cells with damaged or less stringent checkpoints. However, the penalty of proliferation in the absence of DNA repair can be accumulation of mutations in growth-control genes, leading to neoplasia.

To investigate the effect of the loss of BRCA2 function in germ cells in adults, we utilized a line of transgenic mice carrying the human *BRCA2* gene that can rescue the early embryonic lethality of *Brca2*-null mice (*Brca2*$^{-/-}$*; Tg*). These transgenic mice show extremely reduced expression of the human *BRCA2* gene in the testes and ovaries compared with the endogenous mouse *Brca2* gene expression and are consequently sterile. Thus, the *Brca2*$^{-/-}$*; Tg* mice, in effect, are equivalent to a conditional knockout with respect to the gonads and have been used to study the role of BRCA2 in gametogenesis. The localization of BRCA2 on synapsed chromosomes in spermatocytes has suggested its role in meiosis (Chen J et al. *Mol Cell* 2: 317–28, 1998). However, the precise role of BRCA2 in this process and the phenotype associated with the loss of BRCA2 function in gonads is unknown.

The spermaotocytes of *Brca2*$^{-/-}$*; Tg* mice are arrested in the early prophase I stage of meiosis and gradually undergo apoptosis. Immunostaining revealed partial synapsis of chromosomes in the absence of BRCA2, suggesting that the spermatocytes are blocked at the transition from the zygotene to the pachytene stage of prophase I. In addition, we found that the RAD51 repair complex was not appropriately assembled onto meiotic chromosomal axes in the absence of BRCA2 protein. Thus, BRCA2 may be playing a key role in double-strand break repair during meiotic recombination. The fact that BRCA2-deficient murine spermatoctytes undergo apoptosis further supports the hypothesis that the response of cells to damaged DNA is tissue specific. Thus, given the proposed role of BRCA2 in DNA repair in somatic as well as germ cells, cancer predisposition and infertility may represent two different tissue-specific phenotypes resulting from mutations in the gene, more fully revealing its function (Figure 1).

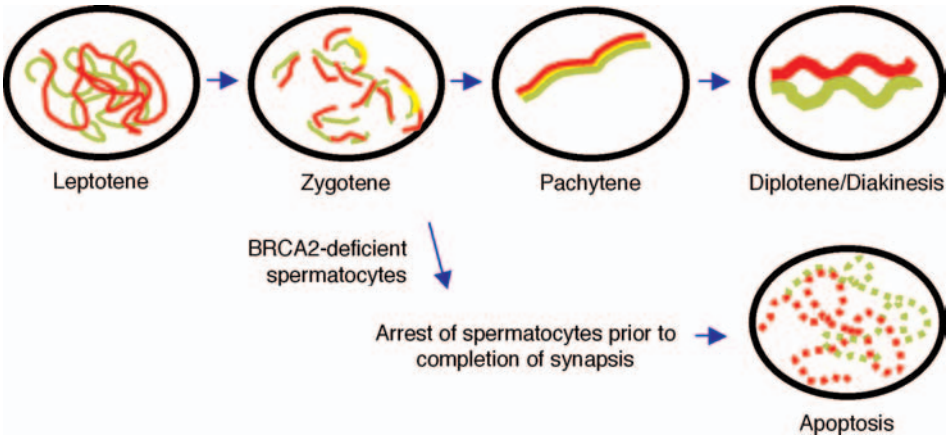

Figure 1. Loss of BRCA2 in spermatocytes results in a block in meiotic progression. Shown here are various stages of normal prophase I of meiosis. During leptotene, there is a gradual condensation of the chromatin, and during zygotene, the homologous chromosomes (shown as green and red lines) start to pair. The synaptonemal complex formation begins (shown as yellow line), and double strand breaks are formed. During pachytene, the condensed homologous chromosomes are tightly paired. This is followed by separation of the homologous chromosomes that remain connected at sites of crossover known as chiasmata. In the absence of BRCA2, there is partial synapsis of chromosomes and the meiotic progression arrests between zygotene and pachytene. The spermatocytes are gradually lost by apoptosis.

In marked contrast to the male meiotic phenotype, BRCA2 protein does not appear to be essential for progression through meiotic prophase in murine oocytes. However, it is required for the normal progression of subsequent stages of meiosis in these cells, as revealed by multiple abnormalities observed in the oocytes undergoing maturation *in vitro*. BRCA2-deficient murine ovaries showed a massive loss of the oocyte pool between postnatal day 2 and 3 weeks. When we superovulated the BRCA2-deficient females, we obtained about 2.5-fold fewer oocytes compared with controls. However, the number of post-implantation embryos from such females was drastically reduced (10-fold less than controls). To explain the marked reduction in the number of embryos obtained, we hypothesize that there could be some abnormalities in female meiosis in these animals. Successful female meiosis is crucial for fertilization and embryo development. To address our hypothesis, oocytes from control and mutant females were examined for their ability to undergo meiotic maturation *in vitro*. Oocytes from the mutant females appeared capable of beginning meiosis, as very few oocytes remained arrested at the germinal vesicle stage after 16 h of culture. However, the meiotic maturation of oocytes from mutant females differed from that of the controls, as few had a normal first polar body, a product of meiosis I.

The meiotic arrest phenotype in BRCA2-deficient mice is sexually dimorphic. Such sexual dimorphism has been observed for many other genes that affect meiosis (Hunt PA et al. *Science* 296: 2181–3, 2002). These phenotypes provide an insight into profound differences between male and female meiosis in mice. Future studies will reveal if the mechanics of meiosis differ between sexes or the meiotic checkpoints vary in spermatocytes and oocytes.

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