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TRANSCRIPTION REGULATION OF ABC DRUG TRANSPORTERS BY MYC ONCOPROTEINS

Supervisor:

Prof. Giuliano DellaValle

Secondary Supervisor:

Prof. Giovanni Perini

PhD Program Coordinator:

Prof. Marialuisa Melli

PhD candidate:

Antonio Porro

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There are two general classes of resistance to anticancer drugs: those that impair delivery of anticancer drugs to tumour cells, and those that arise in the cancer cell itself due to genetic and epigenetic alterations that affect drug sensivity. Impaired drug delivery can result from poor absorption of administered drugs, increased drug metabolism or increased excretion, resulting in lower levels of drug in the blood and reducing drug concentration into the tumour mass [1, 2]. Cancer cells become resistant to anticancer drugs by several mechanisms. One way is to pump drugs out of cells by increasing the activity of efflux pumps, such as ATP-dependent transporters [3]. Alternatively, resistance can also be mediated by reduced drug uptake. Water-soluble drugs are used to bring into the cell by endocytosis or through transporters and carriers, these mechanisms can be alterated in resistant tumor [4, 5].
In cases in which drug accumulation is unchanged, activation of detoxifying proteins can promote drug resistance. The activation of detoxifying systems, such as cytochrome P450mixed-function oxidases, can be induced after exposure to any drug [6]. Cells can also activate mechanisms that repair drug induced DNA damage. Finally, resistance can result from defective apoptotic pathways. This might occur as a result of malignant transformation; for example, in cancer with mutant or non-functional p53. Alternatively, cells might acquire changes in apoptotic pathway during exposure to chemotherapy or changes in cell cycle machinery, which activate checkpoints and prevent initiation of apoptosis.
An important principle in multidrug resistance is that cancer cells are genetically heterogeneous, although the process that results in uncontrolled cell growth in cancer favours clonal expansion, tumor cells that are exposed to chemotherapeutic agents will be selected for their ability to survive and grow in presence of cytotoxic drugs. These cancer cells are likely to be genetically heterogeneous because of the mutator phenotype. So, in any population of cancer cells that is exposed to chemotherapy, more than one mechanism of multidrug resistance can be present. This phenomenon has been called Multifactorial Multidrug Resistance.

CHARACTERIZATION OF THE HUMAN ABC GENE FAMILY

The ATP-binding cassette (ABC) genes represent the largest family of transporter genes and many of these genes are implicated in disease processes and/or drug resistance [7-10]. The prototype ABC protein binds ATP and uses this energy to transport molecules across cell membranes. While hydrophobic compounds are the most common substrates, ABC transporters are able of transporting metal ions, peptides and sugars.
ABC genes are abundant in the genomes of bacteria and archaebacteria where their principal role is in the import of essential molecules [11, 12]. The yeast genome contains 29 ABC genes, and most of these transporters function to move compounds out of the cell or into intacellular organelles [8, 10].
ABC genes are dispersed widely in eukaryotic genomes and are highly conserved between species, indicating that most of these genes have existed since the beginning of eukaryotic evolution. There are 25 ABC genes in E. coli, 29 in S. cerevisiae, 56 in C. Elegans , 56 in Drosophila, 51 in Mouse and 48 in Homo Sapiens [3].
Human ABC genes are localized on 16 different autosomes and 2 genes reside on the X chromosome. Analysis of amino acid sequence alignments of the ATP-binding domains has allowed the ABC genes to be classified into subfamilies. There are seven

ABC genes subfamilies in the human genome. For the most part these subfamilies contain genes that also display considerable identity in the trans-membrane (TM) domains and have identical gene organization, and similar intron localization. Five of these subfamilies are also found in the yeast genome, indicating that these groups were established early in the evolution of eukaryotes, and have been retained. However, the function of ABC genes corresponds poorly to subfamily organization, and often genes in different subfamilies share more similarity in substrate recognition than do genes in the same subfamily [3].

Symbol	Alias	Location	Mouse Location	Expression	Function
ABCA1	ABC1	9q31.1	4 23.1	Ubiquitous	Cholesterol efflux onto HDL
ABCA2	ABC2	9q34	2 12.6	Brain	Drug resistance
ABCA3	ABC3, ABCC	16p13.3		Lung	
ABCA4	ABCR	1p22.1–p21	3 61.8	Rod photoreceptors	N-retinylidiene-PE efflux
ABCA5		17q24	11 69	Muscle, heart, testes	
ABCA6		17q24	11 69	Liver	
ABCA7		19p13.3	10 44	Spleen, thymus	
ABCA8		17q24	11 69	Ovary	
ABCA9		17q24	11 69	Heart	
ABCA10		17q24		Muscle, heart	
ABCA12		2q34		Stomach	
ABCA13		7p11–q11		Low in all tissues	
ABCB1	PGY1, MDR	7p21	5 1.0	Adrenal, kidney, brain	Multidrug resistance
ABCB2	TAP1	6p21	17 18.6	All cells	Peptide transport
ABCB3	TAP2	6p21	17 18.6	All cells	Peptide transport
ABCB4	PGY3	7q21.1	5 1.0	Liver	PC transport
ABCB5		7p14		Ubiquitous	
ABCB6	MTABC3	2q36		Mitochondria	Iron transport
ABCB7	ABC7	Xq12–q13	X 39	Mitochondria	Fe/S cluster transport
ABCB8	MABC1	7q36		Mitochondria	
ABCB9		12q24		Heart, brain	
ABCB10	MTABC2	1q42	8 67	Mitochondria	
ABCB11	SPGP	2q24	2 39	Liver	Bile salt transport
ABCC1	MRP1	16p13.1	16	Lung, testes, PBMC	Drug resistance
ABCC2	MRP2	10q24	19 43	Liver	Organic anion efflux
ABCC3	MRP3	17q21.3		Lung, intestine, liver	Drug resistance
ABCC4	MRP4	13q32		Prostate	Nucleoside transport
ABCC5	MRP5	3q27	16 14	Ubiquitous	Nucleoside transport
ABCC6	MRP6	16p13.1		Kidney, liver	
CFTR	ABCC7	7q31.2	6 3.1	Exocrine tissues	Chloride ion channel
ABCC8	SUR	11p15.1	7 41	Pancreas	Sulfonylurea receptor
ABCC9	SUR2	12p12.1	6 70	Heart, muscle	
ABCC10	MRP7	6p21		Low in all tissues	
ABCC11		16q11–q12		Low in all tissues	
ABCC12		16q11–q12		Low in all tissues	
ABCD1	ALD	Xq28	X 29.5	Peroxisomes	VLCFA transport regulation
ABCD2	ALDL1, ALDR	12q11–q12	15 E–F	Peroxisomes	
ABCD3	PXMP1, PMP70	1p22–p21	3 56.6	Peroxisomes	
ABCD4	PMP69, P70R	14q24.3	12 39	Peroxisomes	
ABCE1	OABP, RNS4I	4q31		Ovary, testes, spleen	Oligoadenylate binding protein
ABCF1	ABC50	6p21.33	17 20.5	Ubiquitous	
ABCF2		7q36	13 40	Ubiquitous	
ABCF3		3q25	16 22	Ubiquitous	
ABCG1	ABC8, White	21q22.3	17 A2–B	Ubiquitous	Cholesterol transport?
ABCG2	ABCP, MXR, BCRP	4q22	6 28–29	Placenta, intestine	Toxin efflux, drug resistance
ABCG4	White2	11q23	5 59	Liver	
ABCG5	White3	2p21	17	Liver, intestine	Sterol transport
ABCG8		2p21	17	Liver, intestine	Sterol transport

PBMC, peripheral blood mononuclear cells; VLCFA, very long chain fatty acids.

Table I. List of human ABC genes, Chromosomal location and Features**.**

ATP-BINDING CASSETTE TRANSPORTERS- PROTEIN STRUCTURE

The ABC genes encode large membrane proteins, they are very difficult to express and purify in quantities sufficient for crystal structure determination. A small number of bacterial NBDs have been crystallized and provide some structural information of the whole protein [13]. In addition, the structure of a few bacterial ABC transporters has been solved [14-16]. Eukaryotic ABC transporters have been even more refractory to structural analysis than bacterial ABC transporters and the first three-dimensional structure for an intact eukaryotic ABC transporter (P-gp) suggests significant differences with the structures of two prokaryotic ABC transporters in the packing of the transmembrane α-helices within this protein family [17].

ABC transporters bind and hydrolyze ATP and use the energy from ATP hydrolysis to pump compounds across the membrane or to flip molecules from the inner to the outer leaflet of the membrane [9, 18, 19]. Genes are classified into the ABC superfamily based on the sequence identity of the ATP-binding domain(s),also known as nucleotide-binding folds (NBFs) [3, 19]. NBFs contain residues that are found in other ATP-binding proteins (the Walker A and B motifs), separated by 90–120 amino acids and an additional element, the signature or C motif, located just in front of the Walker B site [20]. ATP residues are bound by residues from both NBFs. This provides a mechanism where the binding of ATP can induce a substantial structural change in the molecule, sufficient to force the transported compound across the membrane [17]. These proteins also possess two transmembrane (TM) domains composed of 6–11 membrane-spanning α-helices. The transmembrane helices are often at considerable angles to the bilayer or even parallel to the membrane. The functional transporter can either be a single protein with two NBFs and two TM domains (a full transporter) or be a dimer consisting of two half transporters. In vertebrates the ABCA and ABCC subfamilies are composed exclusively of full transporters, the ABCD, ABCG and ABCF subfamily of half transporters and the ABCB subfamily contains both half and full transporters (Fig. 1). The ABCE and ABCF subfamilies consist of proteins with two NBFs and no TM domains. These proteins are not transporters but they are clearly evolutionarily related based on analysis of their NBFs [21].

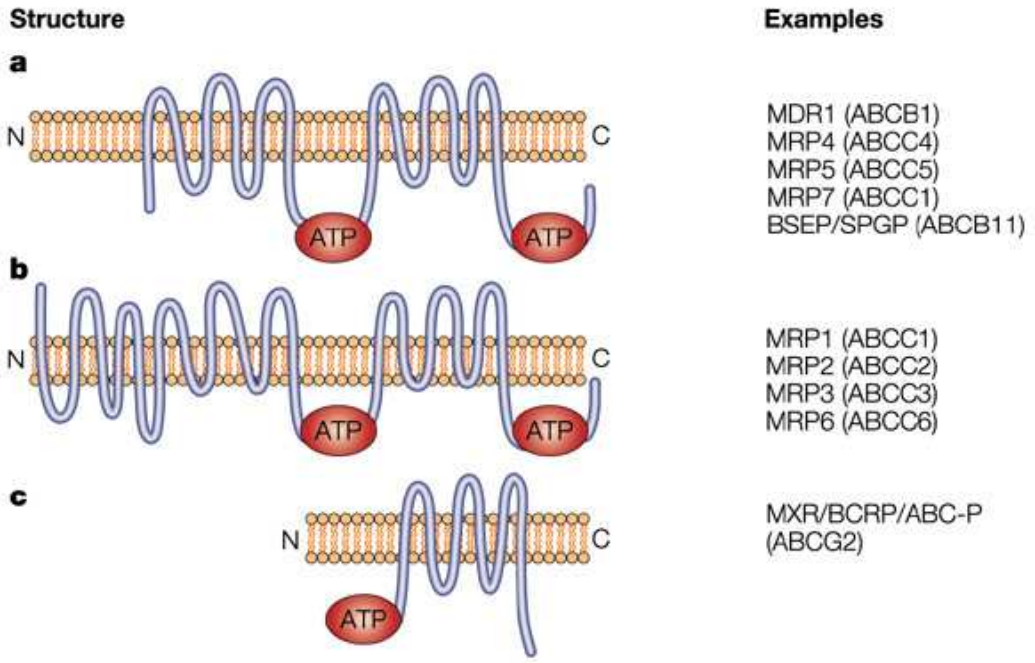

Figure 1. The structure of three categories of ABC transporter

ROLE OF THE NBDs

The overall sequence identity among ABC proteins is low, especially in the TMDs. The presence of more conserved NBDs is consistent with the notion that the varied

functions of the proteins (e.g. ion channels, lipid transporters, peptide transporters) depend on the divergent TMDs; the common feature of nucleotide binding and hydrolysis, however, require the structurally conserved NBDs. Many ABC proteins from lower organisms contain two identical NBDs, and NBD1 and NBD2 of many other ABC proteins, including P-glycoprotein, have very similar sequences and functional properties. In contrast, the two NBDs of other ABC proteins have very different functional roles. Proteins of this kind include those of the MRP subfamily (ABCC) [22].

Both NBDs are required for proper function of mammalian ABC proteins, and a mandatory positive catalytic cooperativity between the NBDs occurs in P-glycoprotein [23-26]. In this protein, hydrolysis occurs at only one NBD per hydrolysis cycle, NBD1 and NBD2 are equivalent, and ATP binds randomly to either NBD. Although there are some asymmetries between the two NBDs of P-glycoprotein, both behave fairly similarly from a functional point of view [22-26]. The current working hypothesis for CFTR channel gating also proposes cooperativity between NBD1 and NBD2. The simplest interpretation of single-channel analysis studies using nucleotide analogs and mutations at NBD1 and/or NBD2 is that the main effects of ATP hydrolysis by NBD1 and NBD2 are to "open" and "close" the channel, respectively[22, 24]. However, recent data point towards a more complex picture that involves a more stable nucleotide interaction and a slower rate of hydrolysis at NBD1, compared with NBD2. The NBDs of SUR also have distinct functions [22]. It has been proposed that SUR ATPase activity resides at NBD2, while NBD1 binds, but does not significantly hydrolyze ATP[22].

The structure of the NBDs is conserved among ABC proteins, independently of the degree of primary-sequence homology. The structure of HisP, the ATP-binding subunit of a histidine permease in *Salmonella thyphimurium*, is shown in Fig. 2A [27, 28] and represents a model for NBDs structure of ABC proteins. Each NBD is formed by two arms. One (Arm I in Fig. 2A) contains the basic core domain, homologous to the F1-ATPase (red α–helices and β–sheets), with the conserved motifs A and B (see Fig. 2B). An antiparallel β-sheet subdomain that interacts with the sugar and base moieties of the nucleotide is also contained in this arm (ABCβ, yellow β-sheets). The basic aspects of binding of the α and β nucleotide phosphates and Mg2+ are conserved between ABC proteins and the F1-ATPase. They share a "Rossman fold" consisting of a central core of β-sheets surrounded by α-helices[28]. Motifs A and B are more than 90 amino acids apart in the primary sequence of ABC proteins (Fig. 2B), but are close to each other in the 3-D structure. Motif A (also called P-loop) is a pyrophosphate-binding site that binds the β and γ phosphates of nucleotide triphosphates. It is located between a β-sheet and an α-helix (red β1 and α1 in Fig. 2B). Most hydrogen bonds are formed between the main-chain nitrogens and the phosphates. Therefore, the residue side chains can vary, consistent with the poor conservation of the primary sequence of the central region of motif A (Gly-**X4**-Gly-Lys-Thr/Ser). The conserved Lys contributes most of the binding energy of the pyrophosphate/motif A interaction. Motif B is in a highly hydrophobic β-sheet that ends in an acidic residue that interacts with Mg2+. The acidic residue is not the catalytic carboxylate, but it is necessary for completion of the hydrolytic cycle[22]. The other arm of the NBDs (Arm II in Fig. 2A) contains a structurally conserved α-helical subdomain (ABCα, green α-helices, Fig. 2). This subdomain contains 3 α-helices and motif C (signature sequence), the most conserved sequence among ABC proteins. It is likely that this subdomain participates in nucleotide binding and also interacts with the TMDs. In the hinge region between the arms, the so-called Gly (approximately equivalent to the γ-phosphate linker labelled in Fig. 2B, purple loop in Fig. 2A and

His loops interact through conserved Gly and His residues with Mg2+ and are involved in the presentation of the hydrolytic water. The molecular mechanism of nucleotide hydrolysis by ABC proteins involves approximation of ATP and Mg2+ and base activation of the attacking water. The activating base is likely a nucleophilic side chain that donates an electron pair to a water molecule, increasing its nucleophilicity. This "activated" water then attacks the bond between the β- and γ-phosphates[22].

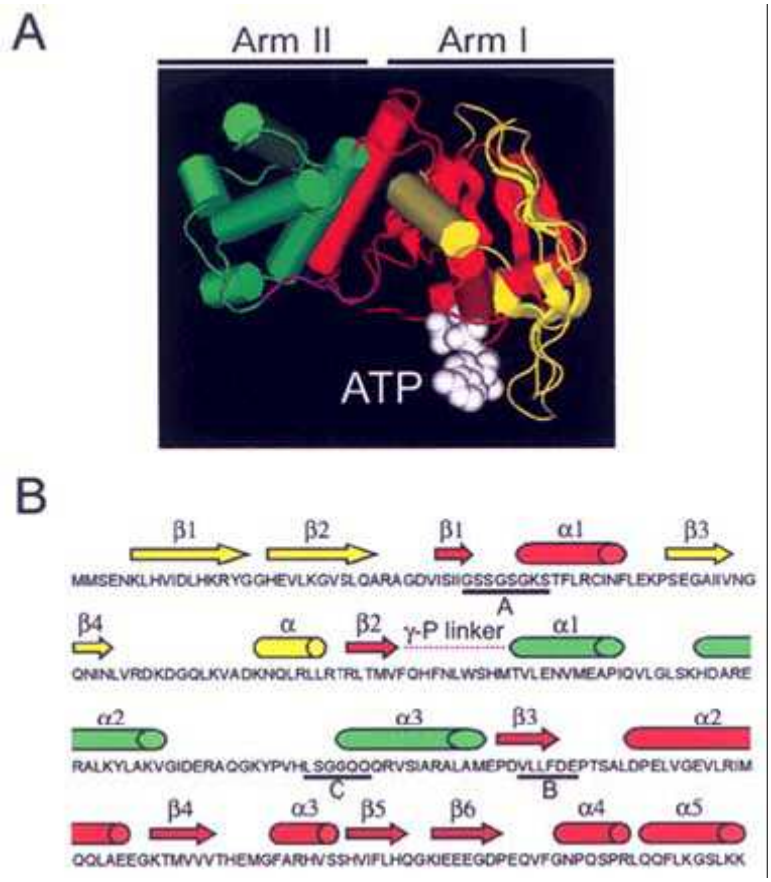

Figure 2. Structure of the nucleotide-binding domains. **a)**. Structure of the ATP-binding subunit of the *Salmonella thyphimurium* histidine permease, HisP. Model NBD structure where α-helices and β-sheets are shown as cylinders and flat arrows, respectively. **b).** Primary and secondary structure of HisP. Cylinders and arrows depict α-helices and β-sheets, respectively. The conserved motifs A, B and C are underlined, and the □-phosphate linker (Gly loop) is marked by a purple dot line. See text for details and color labelling of the subdomains.

Proper function of ABC proteins requires two “normal” NBDs [24-26]. Nucleotide binding to the NBD monomers elicits conformational changes *via* an induced-fit mechanism [29], which do not seem to be responsible for the power stroke that couples substrate transport and ATP hydrolysis [29]. Recent evidence suggests that the two NBDs interact physically, and it has been proposed that this interaction provides the power stroke during the transport cycle. The characteristics of the interaction between NBDs in functional ABC proteins with the complete core domain structure (P-glycoprotein and MRP1) are unknown. However, cysteine crosslinking experiments on P-glycoprotein suggest a Rad50CD-like NBD arrangement, at least at some stages

during the ATP binding/hydrolysis cycle. In apparent contrast with the Rad50CD-like model and most of the available data, recent mutagenesis studies of SUR suggest that motif C is not directly involved in ATP binding. Therefore, additional experiments using functional ABC proteins are needed to resolve the important issue of the nature of the physical interactions of the NBDs during the transport cycle in functional proteins [22].

MOLECULAR MECHANISM OF TRANSPORT

The idea that there is a hydrophobic drug-binding pocket in multidrug-resistance proteins, where hydrophobic substrates bind to the protein without specific interactions can be ruled out based on large differences in affinity for similar compounds (the proteins are poly-specific rather than non-specific). Extensive studies on P-glycoprotein have provided only limited information on the substrates requirements [22]. Hydrophobicity due to the presence of planar aromatic groups is important. A basic nitrogen atom is frequently encountered in good substrates, and a tertiary amine seems associated with high-affinity interaction with P-glycoprotein. Hydrogen bonds play major roles in the protein-drug interaction, with relevant electron donor groups in the drugs that are spatially separated by 2.5 or 4.6 Å. Mutations in P-glycoprotein and MRP1 helix residues that can form hydrogen bonds support their role in the drug-protein interaction [22].

Dimerization of the NBDs of ABC proteins can explain the cooperativity between the NBDs and also provide a general mechanism of coupling between ATP hydrolysis and substrate transport. A speculative model for the catalytic cycle of P-glycoprotein can be presented based on recent biochemical/biophysical and structural data on ABC proteins and NBDs, as well as biochemical and structural studies on P-glycoprotein. The drug can access a high-affinity hydrophobic drug-binding site from the inner leaflet of the membrane or the cytosol, and its binding to the protein produces a conformational change in the NBDs that increases their affinity for ATP [22]. Binding of one ATP to each NBD monomer occurs by interaction with the core and antiparallel subdomains, producing a rotation in the a-helical subdomain that is coupled to the interaction of the γ-phosphate of ATP with the conserved Gly in the γ-phosphate linker (see Fig. 2). ATP binding causes NBD dimerization because of the interaction of the bound ATP with motif C of the other monomer, sandwiching two ATP molecules between the NBDs. Hydrolysis of one ATP produces a conformational change in the TMDs that involves movement of the helices that form the drug-binding pocket [30, 31], with a decrease in drug-binding affinity. The conformational change increases the exposure of the drug to a more hydrophilic environment in the chamber (reducing binding affinity), from where it diffuses to the extracellular solution or is expelled by peristaltic forces exerted by the TMDs. ATP hydrolysis also produces a major decrease in ATP-binding affinity at the other NBD [22, 23], which may result in the release of the non-hydrolyzed ATP. The electrostatic repulsion between the ADP bound to motif A of one monomer and the phosphate bound to motif C of the other monomer destabilizes the dimer state. Phosphate is rapidly released after hydrolysis and ADP is release afterwards (it is the rate limiting step of the hydrolysis cycle) [23]. The drug-binding site, however, remains in a low drug-binding-affinity state and hydrolysis of another ATP is required for resetting the transporter for a new drug transport cycle [22, 23]. Some interesting aspects of the proposed P-glycoprotein catalytic cycle are that hydrolysis occurs at one NBD at any given time, the NBDs are recruited randomly for each hydrolysis cycle, and transport

of one drug is coupled to hydrolysis of two ATP molecules [23]. If the P-glycoprotein catalytic cycle does include hydrolysis of 2 ATPs *per* cycle, as supported by the data and the model proposed by Ambudkar and co-workers [23], it is possible that two NBD association/dissociation events occur *per* transport cycle, one coupled to drug transport and the other to resetting of the transporter for a new cycle (Fig. 3). The power stroke for substrate transport can be the formation or the dissociation of the dimers. Since the NBDs and TMDs are tightly associated, association/dissociation of the NBDs may control the "gate" of the translocation pathway formed by intracellular loops. Tightening of helix packing on the cytoplasmic side could be the key step for substrate efflux (e.g. in P-glycoprotein) and the opposite could be true for substrate influx (Btu). Alternatively, the gate of the chamber on the cytoplasmic side may always be closed and the dimerization of the NBDs could produce conformational changes in the TMDs that reduce drug-binding affinity and also allow for substrate movement from (for efflux) or to (from influx) the inner leaflet of the membrane. Independently of the molecular mechanism, it seems that for P-glycoprotein the power stroke for transport is ATP binding, as opposed to hydrolysis, because the major conformational and functional changes seem to occur at this step. It is possible that this is the case for ABC efflux pumps, while hydrolysis provides the power stroke for the ABC importers by opening the gate of the translocation pathway [22].

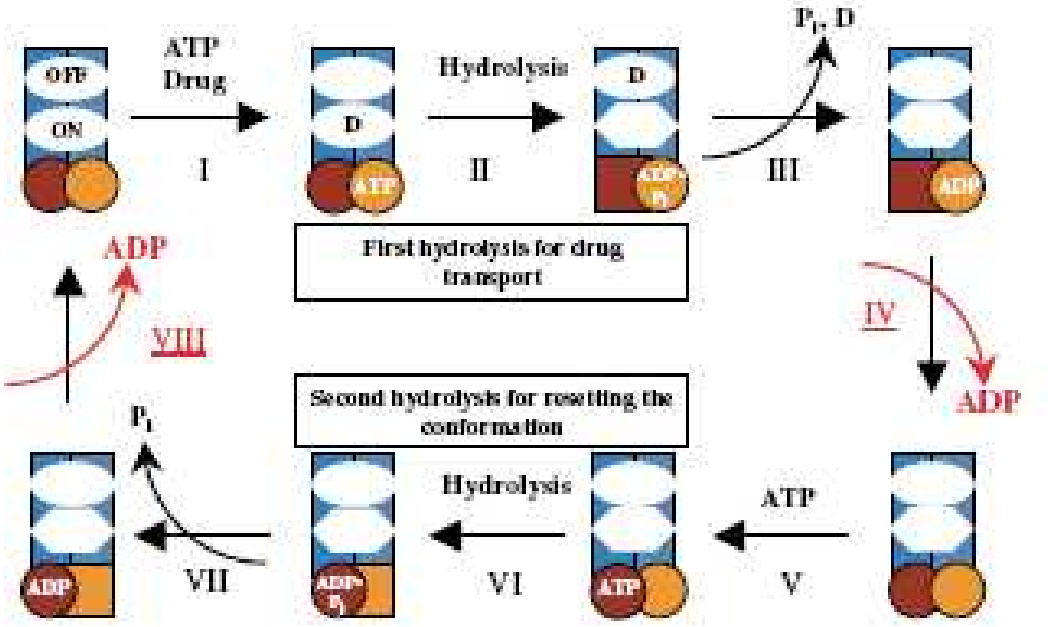

Figure 3. A proposed scheme for the catalytic cycle of P-gp. The ellipses represent the substrate-binding sites: the 'ON' (high affinity) and the 'OFF' (low affinity) site. The hexagon depicts the 'ON' site with reduced affinity for the drug. The green circles represent the ATP sites, and the empty square portrays the ATP site with reduced affinity for nucleotide. Step I: substrate binds to the high-affinity 'ON' site of P-gp, and ATP binds to either of the two ATP sites. Step II: ATP is hydrolysed and the drug is moved to the lower-affinity 'OFF' site. Step III: Pi is released and the drug extruded from P-gp at this step. Step IV: the ADP and Vi dissociate from the complex, the ATP sites revert to the 'high-affinity' state, but affinity for drug substrate continues to be low. Step V: following disassociation of the ADP in step IV, an additional molecule of ATP binds to the alternate ATP site. Step VI: ATP is hydrolysed. Step VII: Pi is released. Step VIII: the disassociation of ADP allows the conformation of P-gp to be restored to its original state (step I), to initiate the next cycle. The ADP release at steps IV and VIII (underlined) appears to be rate limiting in the catalytic cycle. ATP hydrolysis is shown as

being initiated in the C-terminal ATP site; our data suggest that the site of initiation of ATP hydrolysis may be random, but the two sites operate alternately.

OVERVIEW OF HUMAN ABC GENE SUBFAMILIES AND THEIR PHYSIOLOGICAL FUNCTIONS

ABCA SUBFAMILY

This subfamily is composed of 12 full transporters that are split into two subgroups [32]. The first group (*ABCA1–A4, A7, A12, A13*) includes seven genes that map to six different chromosomes. The second group of ABCA genes (*ABCA5–A6, A8–A10*) is organized into a head-to-tail cluster on chromosome 17q24. This gene cluster is also found in the mouse genome.

The expression pattern of the chromosome 17 genes is restricted with *ABCA5* and *ABCA10* expressed in skeletal muscle, *ABCA9* in the heart, *ABCA8* in the ovary, and *ABCA6* in the liver. No diseases map to the corresponding region of the mouse and human genomes, and the functions are as yet uncharacterized. The *ABCA* subfamily genes are dispersed in the genome, except for the cluster on chromosome 17. An alignment of the sequences and phylogenetic analysis demonstrates that the members of the chromosome 17 gene cluster form a distinct subgroup. This is consistent with the genes that have arisen by gene duplication. Analysis of the splice sites of the genes shows that the chromosome 17 gene cluster members each have 38 introns, whereas the other *ABCA* genes have 50 –51 introns. The location of the introns and the size of the exons are highly correlated among the chromosome 17 genes, again supporting a recent duplication. The mouse genome also has a cluster of *ABCA* subfamily genes related to the cluster on chromosome 17. In contrast, there are no such genes in the *Drosophila* or *C. elegans* genomes, suggesting that these genes arose after the separation of vertebrates from insects and worms [32].

ABCA1 controls the extrusion of membrane phospholipids and cholesterol toward specific plasmatic acceptors, the apolipoproteins. It has been proposed that the ABCA-dependent step involves the flux of membrane phospholipids (PL), mostly phosphatidylcoline (PC), toward the lipid-poor nascent apolipoprotein particle, which now can accept cholesterol. The ABCA1-dependent homeostatic control of the lipid content of the membrane dramatically influences the plasticity and fluidity of the membrane itself and, as a result, affects the lateral mobility of membrane proteins and/or their association with membrane domains of special lipid composition. The proposed activity of ABCA1 as a facilitator of the engulfment of apoptotic bodies fits with this view. Indeed, mutations in *ced*-7, a putative *ABCA1* ortholog, in *C. elegans* hampers optimal phagocytosis by precluding the redistribution of phagocyte receptors around the apoptotic particle. The *ABCA4* gene was found to be highly expressed in rod photoreceptors, and maps to the region of chromosome 1p21 containing the gene for the Stargardt disease, a recessive childhood retinal degeneration syndrome. Retinol (vitamin A) derivatives produced in the photoreceptor outer segment disks must be transported to the cytoplasm to be further metabolized and transported out of the cell. ABCA4 is believed to mediate this transport by flipping outwardly modified phosphatidyethanolamine (PE). *Abca4-/-* mice display increased all *trans*-retinaldehyde following light exposure, elevated PE in the rod outer segments (ROS), and accumulation of these compounds. Retinoids stimulate the ATP hydrolysis of the ABCA4 protein in vitro, consistent with a role for these compounds as substrates. It

is of interest in the context of lipid transport to note that photoreceptors represent an exquisite example of membrane dynamics and lipid composition. Indeed, disk membranes are located in the interior of the ROS and arise from evaginations of the ROS plasma membrane. Nascent disks are progressively organized as a discontinuous stacked array of flattened membranous sacs and displaced toward the apical tip as additional new disks are formed. The transition from the base to the tip takes approximately 10 days, and maintains the ROS at constant length. The lipid composition of disk and plasma membranes is dramatically different and suggests a tremendous sorting of lipid constituents at the base of the ROS upon disk biogenesis. During the apical displacement of the disk, their cholesterol content decreases 5-fold, whereas fatty acid and PL composition is virtually unchanged. The loss of cholesterol is thought to take place by its exchange out of the PE-rich disk membrane into the PC-rich plasma membrane; the relative PE/PC ratio being instrumental to favor the movement of cholesterol toward the plasma membrane during the disk life span. The proposed PE flippase activity of ABCA-4 may thus fit well along a delicate sorting pathway of the lipid species across the ROS compartments [32].
The *ABCA2* gene is highly expressed in oligodendrocytes in the brain; the *ABCA7* gene highly expressed in the spleen and thymus. The function of these genes, as well as ABCA12 and ACBA13, is not known, although it is tempting to speculate that they similarly participate in cellular lipid homeostasis in specialized environments. This is supported by the recent findings that both ABCA2 and ABCA7 share with ABCA1 a sterol dependent upregulation [32].

ABCB SUBFAMILY [MULTIDRUG RESISTANCE (MDR)/TAP)]

The ABCB subfamily is composed of four full transporters and seven half transporters, and this is the only human subfamily to have both types of transporters. The *ABCB1* (*MDR/PGY1*) gene was discovered as a protein overexpressed in certain drug-resistant tumor cell lines. Cells that overexpress this protein display MDR and are resistant to or transport a wide variety of hydrophobic compounds including colchicine, doxorubicin, adriamycin, vinblastine, digoxin, saquinivir, and paclitaxel. *ABCB1* is expressed primarily in the liver and blood brain barrier, and is thought to be involved in protecting cells from toxic agents. The gene is duplicated in mice. Animals lacking both genes unfortunately display a very limited phenotype and are still viable and fertile. However, they have been very useful models to identify and characterize other drug resistance genes. Secretion of cholesterol, phospholipids (PL), and other compounds into the bile is critical for normal bile function including the excretion of cholesterol and other sterols and the absorption of fat-soluble vitamins. The ABCB4 and B11 proteins are both located in the liver and participate in the secretion of phophatidylcholine (PC) and bile salts, respectively. Mutations in ABCB4 and ABCB11 are responsible for several forms of progressive familial intrahepatic cholestasis (PFIC). Defects in *ABCB4* are responsible for PFIC3, and are associated with intrahepatic cholestasis of pregnancy. Mutations in the *ABCB11* gene are found in patients with PFIC2. The process of antigen recognition by the class I histocompatibility genes involves the digestion of cellular and foreign proteins into short peptides and their transport into the ER where they form complexes with class I proteins and are expressed on the cell surface. The *ABCB2* and *B3* (TAP) genes are half transporters that form a heterodimer to transport these peptides into the ER. Rare families with defects in these genes display profound immune suppression, as they

lack this essential portion of the immune recognition process. Altered alleles in the TAP genes in the rat are associated with restricted ability to present certain peptides.
The remaining ABCB subfamily half transporters are expressed in the lysosome (*ABCB9*) or the mitochondria (*ABCB6*, *B7*, *B8*, and *B10*). One of the mitochondrial genes (*ABCB7*) is located on the X-chromosome and mutations in this gene are responsible for X-linked sideroblastic anemia and ataxia (XLSA/A) phenotype. The human *ABCB9* gene can complement the yeast ortholog of *ABCB7*, Atm1. This gene plays a role in mitochondrial iron homeostasis and in the biogenesis of cytosolic Fe/S proteins [32].

ABCC SUBFAMILY [CYSTIC FIBROSIS TM CONDUCTANCE REGULATOR (CFTR)/MULTIDRUG RESISTANT PROTEIN (MRP)]

The ABCC subfamily contains 12 full transporters that perform functions in ion transport, toxin secretion, and signal transduction. The MRPs studied thus far, MRP1–5, are all organic anion pumps, but they differ in substrate specificity, tissue distribution, and intracellular location, MRPs come in two structural types, one with 17 transmembrane segments (MRP1, 2, 3, 6), and one with 12 (MRP4, 5, 7, 8). Cystic fibrosis (CF) is an inherited multisystemic disorder characterized by anormalities in exocrine gland function consequent to loss of function of the CFTR transporter (ABCC7). The CFTR protein is unique among ABC proteins in that it is a cAMP-regulated chloride ion channel [33].
The *ABCC8* gene was identified as the locus for familial persistent hyperinsulinemic hypoglycemia of infancy, an autosomal recessive disorder characterized by unregulated insulin secretion. Subsequent work demonstrated that the *ABCC8* gene is a high affinity receptor for the drug sulfonylurea. Sulfonylureas are widely used to increase insulin secretion in patients with non-insulin-dependent diabetes. These drugs bind to the ABCC8 and the closely related ABCC9 protein, and inhibit the KIR6 potassium channel [32]. The remaining ABCC genes are nine MRP-related genes. *ABCC1 (MRP1)* was identified as a multidrug resistance gene and demonstrated to transport glutathione conjugates of many toxic compounds. MRP1 is a prototype GS-X pump and a remarkably versatile one. It transports a variety of drugs conjugated to GSH, to sulfate or to glucuronate, as well as anionic drugs and dyes, but also neutral/basic amphipathic drugs and even oxyanions. The oxyanions arsenite and antimonite and the neutral/basic drugs are co-transported with GSH. Notwithstanding this enormous range of substrates transported, MRP1 is not indiscriminate [34]. Similar to ABCB1, ABCC1 transports and confers resistance to a wide variety of toxic substrates, but is not essential for growth or development. ABCC1 can also transport leukotriene C4, a potent chemotactic factor controlling dendritic cell migration from peripheral tissues to lymph nodes [32]. In the brain, MRP1 has an important function in the “cleansing” of toxic compounds from cerebrospinal fluid (CSF). These cells normally contain high levels of MRP1 in their basolateral membrane; an absence of *MRP1* causes etoposide levels to increase ten-fold in the CSF after intravenous administration of the drug. Obviously, the body needs a basolateral transporter to protect sperm or CSF. P-gp in the apical membrane would pump drug into the cavity rather than protect its content.
MRP2 and MRP1 have about the same size and putative membrane topology, and they both transport a similar large range of organic anions. However, the tissue distribution of MRP2 is much more restricted than that of MRP1, and MRP2 is located in the apical membrane of epithelial cells, whereas MRP1 is basolateral.

MRP2 has an important function in the biliary excretion of endogenous metabolites, such as glucuronosyl-bilirubin, as well as many exogenous compounds. *MRP2* is expressed not only in the liver but also in the kidney and the intestine of rats and humans. There is considerable species difference in the level of expression in these two organs compared to the liver. The most extreme example is in rabbits, in which expression in kidney and intestine is higher than in the liver. Intestinal MRP2 excretes organic anions into the gut and plays a role in reducing the oral availability of food-derived carcinogens. Mutations in the *MRP2* gene cause the Dubin-Johnson syndrome. These patients suffer from an inherited conjugated hyperbilirubinemia, which indicates that bilirubin can enter the hepatocytes and is conjugated with glucuronate, but is not secreted into bile. Because of the similarity in the substrates transported by MRP1 and 2, one would expect cells transfected with the *MRP2* gene constructs to become resistant to the same range of anticancer drugs as *MRP1* transfectants. It has been technically difficult to verify this prediction because MRP2 is not routed efficiently to the plasma membrane in most transfected cells. Whether MRP2 contributes to anticancer drug resistance in patients remains to be seen. MRP2 has been detected in renal, lung, gastric, colorectal, and hepatocellular carcinomas, but no correlation between MRP2 and clinical resistance has emerged [32].

MRP3 is an organic anion transporter, basolateral like MRP1 [35], and prominently present in liver, gut, and kidney like MRP2. The strong upregulation of MRP3 in the liver under some cholestatic conditions and the ability of MRP3 to transport some bile salts [36] have led to speculations that MRP3 might play a role in the enterohepatic recycling of bile salts and in the removal of toxic organic anions from the hepatocyte under cholestatic conditions [35]. MRP3 differs from MRP1 and 2 in that it appears unable to transport GSH [35]. This may explain why cells transfected with MRP3 gene constructs are not resistant to most of the anticancer drugs that are probably cotransported with GSH by MRP1/2. The only exceptions are the epipodophyllotoxins etoposide and teniposide [34, 35]. MRP3-mediated resistance against these drugs does not require intracellular GSH, and etoposide appears to be transported by MRP3 in unmodified form. MRP3 is present in cancer cell lines from many tissues [37], but initial studies on MRP3 in a panel of drug-resistant cancer cell lines did not turn up any association between MRP3 levels and resistance [37]. MRP3 levels are high in the adrenal cortex [38]. Given the high affinity of MRP3 for estradiol-17β-glucuronide, it is therefore possible that MRP3 contributes to the transport of endogenous steroid conjugates [34]. Glucoronide and sulphate conjugates of bile salts are substrates of both MRP1 and MRP3, but MRP3 also mediates transfer of monovalent bile salts including glycocholate [39]. Glucoronide conjugates (e.g.,E217βG) seem to be preferentially transported by MRP3 compared with GSH conjugates such as 2,4-dinitrophenyl *S*-glutathione (DNP-SG) and leukotriene C4 (LTC4) [39, 40].

MRP4 and MRP5 are both organic anion pumps, but they have the interesting ability to transport cyclic nucleotides and nucleotide analogs, a class of organic anions apparently not transported by MRP1–3 or 6. The transport of nucleotide analogs by MRP4 and 5 can result in resistance to clinically used base, nucleoside, and nucleotide analogs, at least in transfected cells that highly overproduce MRP4 or 5. The rate of cyclic nucleotide transport by these transporters is low and the physiological role of this transport remains to be defined.

Initial studies on MRP5 showed that this protein is an organic anion pump, able to transport acidic organic dyes, *S*-(2,4-dinitrophenyl)glutathione, GS-DNP, and GSH, and inhibited by sulfinpyrazone. However, substantial drug resistance in *MRP5*-transfected cells was found only for 6-mercaptopurine (6MP) and thioguanine (TG),

two purine bases that are definitely not acidic [34]. This paradoxical result was rationalized when Schuetz et al. [41] discovered that a cell line selected for resistance against PMEA, an adenine nucleotide analog, highly overexpressed the *MRP4* gene. This suggested that MRP4 and 5 are nucleotide transporters and that the resistance of MRP5 cells to thiopurines was a result of conversion of the bases into the corresponding nucleotides and extrusion of the nucleotides from the cell by MRP5. Whereas MRP4 appears to prefer methylated thioIMP, MRP5 prefers the unmethylated thionucleotides. There is no indication that MRP5 can transport nucleoside di- or triphosphate analogs.

Cells with high concentrations of MRP4 are highly resistant to PMEA and AZT and much less resistant to other nucleoside analogs used in antiviral therapy, such as lamivudine, ddC, and d4T [41]. MRP4, but not MRP5, confers resistance to short-term incubation with high concentrations of MTX. Like MRP4, MRP5 can transport nucleotide analogs with a normal pyrimidine ring [34]. The list of substrates transported by MRP4 and 5 was substantially broadened by vesicular transport studies. Jedlitschky et al. [42] discovered that MRP5 can transport cyclic GMP and AMP (cGMP and cAMP), and Hopper et al. [43] recently found this for MRP4 as well. The affinity for cGMP is higher than for cAMP. There is no indication that MRP4 or 5 can use standard 5'- or 3'-mononucleotides as substrate. Estradiol-17β-glucuronide is transported relatively well by MRP4. Interestingly, the cGMP transport by MRP5 is efficiently inhibited by the inhibitors of cGMP phosphodiesterase, sildenafil (Viagra), trequinsin, and zaprinast. The tissue distribution of MRP4 and MRP5 is still not well known. Recent studies suggest that *MRP4* is more widely expressed than initially thought, with the highest levels in kidney and prostate. Analysis of tissue RNA suggests that *MRP5* is ubiquitously expressed. The highest levels are found in skeletal muscle and brain. All attempts to generate antibodies that allow the localization of MRP5 in tissues with anti-MRP5 antibodies have failed thus far. This is presumably because the expression levels are too low, as these antibodies readily detect MRP5 in transfected cells. On Western blots MRP5 can be detected in human and murine erythrocytes, which might explain the observed cGMP transport in these cells, although this needs verification using red cells from *Mrp5* (-/-) mice. The physiological functions and possible role in drug resistance of MRP4 and 5 remain to be defined. Obviously, the discovery that these pumps can transport cyclic nucleotides, notably cGMP, has raised the question of whether MRP4/5 can affect the signal transduction role of cGMP by removing it from the cell, which would supplement the degradation by phosphodiesterases. There is also evidence for an extracellular signaling role for cGMP in kidney and several other tissues, and MRP4/5 might be involved. Any role that MRP4/5 may have in drug resistance is also under investigation. As nucleobase and nucleoside analogs are used extensively in anticancer and antiviral therapies, there is potential for MRP4/5 to mediate resistance to these compounds. 6MP and methotrexate are both used in the treatment of childhood leukemias and MRP4 is the only drug transporter known thus far that can transport both drugs [34]. Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and is also able to confer significant resistance in vitro to the topoisomerase I poison irinotecan and its active metabolite SN-38. This drug is approved for treatment of colon and lung cancers and shows promising activity against a number of other cancers including cervical, ovarian carcinomas, and also neuroblastoma [44]. No human disease has been associated with alterations in MRP5, and the Mrp5 knock out mouse, has no obvious phenotype. It is possible, however, that the overlapping substrate specificities of MRP5 and MRP4 (and possibly MRP8 and 9) may hide the physiological function of MRP5.

Human *MRP6* is mainly expressed in liver and kidney, like *Mrp6* (*MLP-1*), its rat homolog, but low RNA levels have also been detected in other tissues. The substrate specificity of MRP6 is still a mystery. Madon et al. [45] tested a series of typical MRP substrates in vesicular transport studies and found transport only of BQ-123, an anionic cyclopentapeptide and an antagonist for the endothelin A receptor. Endothelin-1 itself was transported by MRP2, but not by MRP6. These results suggest that MRP6 could be a highly selective pump for organic anions. Amplification of the 3' part of the *MRP6* gene was found in leukemia cells selected for anthracycline (epirubicin) resistance [34]. The anthracycline resistance was initially thought to be due to a new resistance determinant, called the anthracycline resistance gene, *ARA*. Subsequent work has shown, however, that the epirubicin resistance of cell lines with *ARA* gene amplification can be explained by co-amplification of the *MRP1* gene together with the 3' half of the adjacent *MRP6* gene [34]. There is no indication that the *MRP6* gene is ever associated with anticancer drug resistance. How defects in MRP6 cause pseudoxanthoma elasticum, a heritable disorder characterized by calcification of elastic fibers in skin, arteries, and retina, is unclear. Why the loss of a highly specialized pump located in the basolateral membrane of liver and kidney cells would lead to such a generalized connective tissue disease is hard to explain. Speculations include indirect effects on Ca2+ metabolism or elastic fiber assembly through excretion of cytokine-like organic anionic peptides [34].

ABCD SUBFAMILY [ADRENOLEUKODYSTROPHY (ALD)]

This subfamily contains 4 genes that encode half transporters expressed exclusively in the peroxisome. ABCD1– 4, which are also called ALDP, ALDR, PMP70, and PMP69, respectively. Interaction between these proteins was demonstrated by co-immunoprecipitation and yeast two-hybrid assays [34]. One of the genes, *ABCD1*, is responsible for the X-linked form of ALD, a disorder characterized by neurodegeneration and adrenal deficiency, typically initiating in late childhood. The presentation of ALD is highly variable with adrenomyeloneuropathy, childhood ALD, and adult onset forms. However, there is no correlation between the phenotype of ALD and the genotype at the *ABCD1* locus. Cells from ALD patients are characterized by an accumulation of unbranched saturated fatty acids, but the exact role of ABCD1 in this process has yet to be elucidated. The functions of the other ABCD family genes have also not been worked out, but the marked sequence similarity (especially for *ALDP-ABCD2*) suggest that they may exert related functions in fatty acid metabolism. The in vitro demonstration of homo- or heterodimerization of the product of ABCD1 with either ALDRP or PMP70 suggest that different peroxisomal half transporter heterodimer combinations are involved in the import of specific fatty acids or other substrates. *ABCD* genes are under complex regulation at the transcriptional level, and being very tightly linked to cell lipid metabolism, it is not surprising that they share with the ABCA and ABCG subclasses the sensitivity to the peroxisome proliferator-activated receptor and retinoid X receptor family of nuclear receptors [32].

ABCE [OLIGOADENYLATE BINDING PROTEIN (OABP) AND ABCF (GCN20)-NONMEMBRANE ABC PROTEINS] SUBFAMILIES

The ABCE and ABCF subfamilies are composed of genes that have ATP-binding domains that are closely related to those of the other ABC transporters, but these genes do not encode any TM domains. The ABCE subfamily contains a single member, the OABP, ABCE1. This protein recognizes oligoadenylate produced in response to certain viral infections [32]. The *ABCE1* gene is the most conserved member of the ABC gene family and is one of the most conserved genes in vertebrate and archaeal genomes [46]. This fact alone suggests that the gene plays an essential role in biology that is common between archae and eukaryotes. In addition, null mutations in the gene are homozygous lethal in every organism that has been examined. *ABCE1* is essential in *Xenopus* and that suppression of translation or splicing with morpholino oligonucleotides results in the cessation of growth of the embryo during gastrulation, a period when the germ layers of the embryo are formed and the body plan of the mature organism is established.

ABCE1 mRNA was detectable by reverse transcription-PCR in oocytes, and growth cessation presumably occurs at the point at which most of the maternal protein has degraded. The ABCE1 protein was originally identified due to an interaction with and inhibition of RNase L, a nuclease induced by interferon [47]. However, RNase L is not found outside of vertebrates, indicating that

ABCE1 has alternate functions. The identification of the role of the RLI/ABCE1 protein in ribosome biogenesis and in assembly of the pre-initiation complex of the ribosome in *S. cerevisiae* provides a function that is both essential and universal to eukaryotes [48]. Therefore this is likely to be the original role of ABCE1 and the protein has adapted interaction with RNase L as a secondary function. The mammalian ribosome is substantially different from the yeast ribosome. For example the initiation complex compnent eIF3 has 14 subunits in mammals and only 6 in yeast. Therefore the importance of ABCE1 in mammalian protein initiation required experimental evidence.

The important role of ABCE1 in protein synthesis is extended by the data showing that the protein is essential in *in vitro* and *in vivo* translation of mammalian proteins. Antisera to ABCE1 block *in vitro* translation of mRNA in rabbit reticulocyte lysates but not of poly(U) molecules that can be translated independent of initiation factors. As in yeast, ABCE1 interacts with the eukaryotic initiation factors eIF5 and eIF2 components of the pre-initiation complex. Inhibition of ABCE1 in human cells results in dramatic inhibition of growth, reduction in the amount of large polysomes, and incorporation of labeled amino acids into newly synthesized protein. This is consistent with the results in yeast and supports a critical role for ABCE1 in the initiation of translation. The inhibition of ABCE1 could have therapeutic applications. Because the protein is essential to most or all eukaryotes, specific inhibitors could be used in the treatment of pathogens. For example, inhibitors specific to plasmodia, fungi, and/or protozoan parasites could be used to inhibit such organisms as they infect human or other animals. We have shown here that ABCE1 inhibitors efficiently suppress the growth of human tumor cells. It is known that tumor cells have a high capacity for protein translation, and proteins involved in translation such as S6 kinase, mTOR, and 4E-BP1 are molecular targets for cancer therapy. It is possible that cancer cells are more sensitive to inhibition of protein translation through ABCE1 than are normal cells. Last, ABCE1 is required for the assembly of HIV-1 and other lentiviruses. Drugs that interfere with the HIV/ABCE1 interaction could be used as antiretroviral agents. The ABCE1 protein is unusual in

containing a Fe-S cluster binding site. It has long been known that Fe-S clusters are assembled in the mitochondria, and this process is essential to the cell. ABCE1 clearly represents one essential Fe-S containing protein [46].
The ABCF genes each have a pair of NBF, and the best characterized member is the *S. cerevisiae* GCN20 gene. GCN20 is involved in the activation of the eIF-2 alpha kinase. A human homolog, ABCF1 is part of the ribosome complex and may play a similar role [32].

ABCG (WHITE) SUBFAMILY

The human ABCG subfamily contains six half transporters that have an NBF at the N-terminus and a TM domain at the C-terminus: the reverse of the orientation of all other ABC genes. The *Drosophila White* locus was the first gene located by genetic mapping. The white protein forms a heterodimer with either of two other ABCG-related proteins, brown and scarlet, to transport guanine and tryptophan in the eye cells of the fly. These molecules are precursors of the fly eye pigments. Surprisingly, there are only 5 *ABCG* genes in the human genome, whereas there are 15 in the *Drosophila* genome and 10 in yeast. Evolutionary analysis of the yeast genes shows that nearly all of them diverged a long time ago. This is also evident in analysis of the position of the introns that shows that they are not conserved among the genes [32]. The only exception is the *ABCG1* and *ABCG4* genes. This pair is closely related both in amino acid sequence and in having nearly identical intron location. *ABCG1* is highly expressed in macrophages and is induced by cholesterol. *ABCG4* is highly expressed in the brain. It will be interesting to see if these genes have related functions. The *ABCG5* and *ABCG8* genes are located head-to-head on the human chromosome 2p15-p16, separated by a region of 200 bp. The genes are both mutated in families with sitosterolemia, a disorder characterized by defective transport of plant and fish sterols and cholesterol. Sitosterolemia patients display deficient sterol secretion from the intestine and the liver. This genetic evidence indicates that the two half transporters form a functional heterodimer. This is supported by the finding that the two genes are coordinately regulated by cholesterol. Perplexingly, the *ABCG5* gene is principally mutated in Asians; the *ABCG8* gene in Caucasians. This suggests that the proteins may form both hetero- and homodimers to transport the wide range of dietary sterols (compesterol, stigmasterol, avenosterol, sitosterol, cholesterol) encountered in the diet. The mammalian *ABCG1* gene is also induced by cholesterol and is involved in cholesterol transport regulation. The analysis of cell lines selected for high level resistance to mitoxantrone that do not overexpress *ABCB1* or *ABCC1* were instrumental in the identification of the *ABCG2* (*ABCP*, *MXR1*, *BCRP*) gene as a multidrug transporter. *ABCG2* can use anthracycline anticancer drugs, as well as topotecan, mitoxantrone, or doxorubicin as substrates. The *ABCG2* gene is either amplified or rearranged by chromosomal translocations in resistant cell lines. Transfection of *ABCG2* into cells confers resistance, consistent with its functioning as a homodimer. ABCG2 can also transport several dyes (rhodamine and Hoechst 33,462), and the gene is highly expressed in a subpopulation of hematopoetic stem cells (side population) but not in mature blood cells. The normal function of ABCG2 is not known; however, it is highly expressed in placental trophoblast cells, suggesting that it may pump toxic metabolites from the fetal to the maternal blood supply. The *Abcg3* gene is so far only found in the mouse and other rodent genomes. The gene is expressed in the spleen and thymus and has an ATP-binding domain that is missing several conserved residues in the Walker A and Signal domains [32].

ABC GENES AND HUMAN GENETIC DISEASE

Many ABC genes were originally discovered during the positional cloning of human genetic disease genes. To date, 14 ABC genes have been linked to disorders displaying Mendelian inheritance. As expected from the diverse functional roles of ABC genes, the genetic deficiencies that they cause also vary widely. Because ABC genes typically encode structural proteins, all of the disorders are recessive, and are attributable to a severe reduction or lack of function of the protein. Heterozygous variants in ABC gene mutations, however, are being implicated in the susceptibility to specific complex disorders [3].

CYSTIC FIBROSIS AND CFTR (ABCC7)

Cystic fibrosis is the most common fatal childhood disease in Caucasian populations, reaching frequencies ranging from 1: 900 to 1:2500. This corresponds to a carrier frequency of 1:15–1:25. The disease is much less common in African and Asian populations, where carrier frequencies of 1:100 to 1:200 have been estimated. The disease frequency correlates with the frequency of the major allele of the CF gene, a deletion of three base pairs (ΔF508). At least two other populations, however, have high frequency CF alleles. The W1282X allele is found on 51% of the alleles in the Ashkenazi Jewish population and the 1677delTA allele has been found at a high frequency in Georgians and is also present at elevated level in Turkish and Bulgarian populations. This has led several groups to hypothesize that these alleles arose through selection of an advantageous phenotype in the heterozygotes. It is through CFTR that some bacterial toxins such as cholera and *E. coli* cause increased fluid flow in the intestine and result in diarrhea. Therefore, several researchers have proposed that the CF mutations have been selected for in response to these diseases. This hypothesis is supported by studies showing that CF homozygotes fail to secrete chloride ions in response to a variety of stimulants, and a study in mice in which heterozygous null animals showed reduced intestinal fluid secretion in response to cholera toxin. CFTR is also the receptor for *Salmonella typhimurium* and implication in the innate immunity to *Pseudomonas aeruginosa*. Patients with two severe CFTR alleles like ΔF508 typically display severe diseases with inadequate secretion of pancreatic enzymes leading to nutritional deficiencies, bacterial infections of the lung, and obstruction of the vas deferens, leading to male infertility. Patients with at least one partially functional allele display enough residual pancreatic function to avoid the major nutritional and intestinal deficiencies and subjects with very mild alleles display only congenital absence of the vas deferens, a genetic cause of male infertility, with none of the other symptoms of CF. Recently, heterozygotes of CF mutations have been found to have an increased frequency of pancreatitis and bronchiectasis. Therefore, there is a spectrum of severity in the phe-notypes caused by this gene that is inversely related to the level of CFTR activity. Clearly, other modifying genes and the environment also affect disease severity, particularly the pulmonary phenotypes [3].

ADRENOLEUKODYSTROPHY/ABCD1

Adrenoleukodystrophy (ALD) is an X-linked recessive disorder characterized by neurodegenerative phenotypes with onset typically in late childhood. Adrenal

deficiency commonly occurs and the presentation of ALD is highly variable. AMN, childhood ALD and adult onset forms are recognized, but there is no apparent correlation to *ABCD1* alleles. ALD patients have an accumulation of unbranched, saturated fatty acids with a chain length of 24–30 carbons, in the cholesterol esters of the brain and in adrenal cortex. The ALD protein is located in the peroxisome, where it is believed to be involved in the transport of very long chain fatty acids [3].

FAMILIAL PERSISTENT HYPERINSULINEMIC HYPOGLICEMIA OF INFANCY /ABCC8

The *ABCC8* gene is a high-affinity receptor for the drug sulfonylurea. Sulfonylureas are a class of drugs widely used to increase insulin secretion in patients with non-insulin-dependent diabetes. These drugs bind to the ABCC8 protein and inhibit an associated potassium channel KIR6.2 (KCNJ11). Familial persistent hyperinsulinemic hypoglycaemia of infancy is an autosomal recessive disorder in which subjects display unregulated insulin secretion. The disease was mapped to 11p15-p14 by linkage analysis, and mutations in the *ABCC8* gene are found in PHHI families. Multiple studies have reported association of the E23K variant of Kir6.2 with risk of type 2 diabetes. However, this variant has a very strong allelic association with the A1369S variant in the *ABCC8* gene. Thus the association cannot be ascribed to either gene and may be a compound effect of both variants [21].

BILE SALT TRANSPORT DISORDER

Several ABC transporters are specifically expressed in the liver, have a role in the secretion of components of the bile, and are responsible for several forms of progressive familial intrahepatic cholestasis (PFIC). PFICs are a heterogeneous group of autosomal recessive liver disorders, characterized by early onset of cholestasis that leads to liver cirrhosis and failure before adulthood. The *ABCB4* (PGY3) gene transports phosphatidylcholine across the canalicular membrane of hepatocytes. Mutations in this gene cause PFIC3 and are associated with intrahepatic cholestasis of pregnancy. The rat *Abcc2/Mrp2* gene was found to have a frame-shift mutation in the strain defective in canalicular multispecific organic anion transport, the TR- rat. The TR- rat is an animal model of Dubin-Johnson syndrome and mutations in *ABCC2* have been identified in Dubin- Johnson syndrome patients. The ABCC2 protein is expressed on the canalicular side of the hepatocyte and mediates organic anion transport. The *ABCB11/BSEP* gene was originally identified based on homology to *ABCB1*. *ABCB11* is highly expressed on the liver canalicular membrane and has been shown to be the major bile salt export pump. Mutations in *ABCB11* are found in patients with PFIC2 [3].

RETINAL DEGENERATION/ABCA4

The *ABCA4/ABCR* gene is expressed exclusively in photoreceptors where it transports retinol (vitamin A) derivatives from the photoreceptor outer segment disks into the cytoplasm [49]. The chromophore of a visual pigment rhodopsin, retinal, or conjugates with phospholipids are the likely substrates for ABCA4, as they stimulate the ATP hydrolysis of the protein. Mice lacking *Abca4* show increased all-trans-

retinaldehyde (all-trans-RAL) following light exposure, elevated phosphatidylethanolamine (PE) in outer segments, accumulation of the protonated Schiff base complex of all-trans-RAL and PE (N-retinylidene-PE), and striking deposition of a major lipofuscin fluorophore (A2-E) in retinal pigment epithelium (RPE). These data suggest that ABCR is an outwardly directed flippase for N-retinylidene-PE. Mutations in the *ABCA4* gene have been associated with multiple eye disorders [50]. A complete loss of ABCA4 function leads to retinitis pigmentosa, whereas patients with at least one missense allele have Startgardt disease (STGD). STGD is characterized by juvenile to early adult onset macular dystrophy with loss of central vision. *ABCA4* mutation carriers are also increased in frequency in age-related macular degeneration (AMD) patients. AMD patients display a variety of phenotypic features, including the loss of central vision after the age of 60. The causes of this complex trait are poorly understood, but a combination of genetic and environmental factors have a role. The abnormal accumulation of retinoids, caused by ABCA4 deficiency has been postulated to be one mechanism by which this process could be initiated. Defects in ABCA4 lead to an accumulation of retinal derivatives in the retinal pigment epithelium behind the retina [3].

MITOCHONDRIAL IRON HOMEOSTASIS/ABCB7

Several half transporters of the MDR/TAP subfamily have been localized to the inner membrane of the mitochondria. The yeast ortholog of *ABCB7*, Atm1, has been implicated in mitochondrial iron homeostasis, as a transporter in the biogenesis of cytosolic Fe/S proteins. Two distinct missense mutations in *ABCB7* are associated with the X-linked sideroblastic anemia and ataxia (XLSA/A) phenotype. Three more half transporters from this subfamily, ABCB6, ABCB8 and ABCB10 have also been localized to mitochondria [3].

STEROL TRANSPORT DEFICIENCIES

Tangier disease is characterized by deficient efflux of lipids from peripheral cells, such as macrophages, and a very low level of HDL. The disease is caused by alterations in the *ABCA1* gene, implicating this protein in the pathway of removal of cholesterol and phospholipids onto HDL. Patients with hypolipidemia have also been described that are heterozygous for *ABCA1* mutations, suggesting that ABCA1 variations may have a role in regulating the level of HDLs in the blood. Subsequently, the sterol-dependent regulation of *ABCA1* expression was shown. Current models for ABCA1 function place it at the plasma membrane where it mediates the transfer of phospholipid and cholesterol onto lipid-poor apolipoproteins to form nascent HDL particles. The ABCA1-mediated efflux of cholesterol is regulated by nuclear hormone receptors, such as oxysterol receptors (LXRs) and the bile acid receptor (FXR), as heterodimers with retinoid X receptors (RXRs). ABCA1 also plays a role in the engulfment of apoptotic bodies. Furthermore, the *ced-7* gene, an ABCA1 ortholog in *C. elegans*, plays a role in phagocytosis by precluding the redistribution of phagocyte receptors around the apoptotic particle. Recently, two half-transporter genes, *ABCG5* and *ABCG8* were characterized, located head-to-head on the human chromosome 2p15-p16, and regulated by the same promoter. These genes are both mutated in families with sitosterolemia, a disorder characterized by defective transport of plant and fish sterols and cholesterol. Most likely, the two half-

transporters form a functional heterodimer. The *ABCG1* gene is also regulated by cholesterol and *ABCG4* is highly expressed in the liver, suggesting that these two genes may also be involved in cholesterol transport [3].

LAMELLAR ICHTYOSIS TYPE 2 (LI2)/ABCA12

Lamellar ichthyosis type 2 is a genetically heterogeneous skin disorder characterized by large, dark, pigmented scales. The disease was mapped to chromosome 2q33–35, the region where *ABCA12* is located. Mutations in *ABCA12* were identified in several LI2 families. It is likely that ABCA12 plays a role in lipid secretion or membrane organization in the developing skin. The *ABCA12* gene is also expressed in the stomach, indicating that it may play a role in mucous secretion [21].

SURFACTANT DEFICIENCY/ABCA3

Respiratory distress syndrome is an important cause of neonatal mortality and morbidity and is often caused by a deficiency in lung surfactants. Surfactant forms a lipid rich monolayer that coats the pulmonary airways and is essential for the inflation of the lung. Surfactant is produced and secreted by alveolar type II cells and consists of lipids, cholesterol, and specialized proteins. The *ABCA3* gene is expressed in alveolar type II cells, and the protein is localized to lamellar bodies. Mutations in the *ABCA3* gene are an important cause of this disease, and patients display abnormal surfactant, elevated surface tension, and abnormal lamellar bodies. Although typically fatal, mild cases have been identified and associated with missense mutations in the *ABCA3* gene [21].

IVERMECTIN SENSITIVITY/ABCB1

Ivermectin is a highly useful drug that is effective against a variety of invertebrates, including helminthes, Onchocerca volvulus (the worm causing river blindness), and mites (including those causing scabies). Ivermectin is in wide use in both veterinary and human medicine. Collie dogs frequently display sensitivity to the drug, as do mice that are *Abcb1a* -/-. It was found that collies have a 4bp deletion in the gene and that at least nine other breeds carry this mutation. ABCB1 is a drug transporter that is expressed in many tissues, but plays a particularly important role in the blood–brain barrier. ABCB1 mutant collies are also sensitive to a number of other drugs, including doramectin, loperamide, and several anticancer drugs [21].

IMMUNE DEFICIENCY/TAP1 AND TAP2

The *TAP1/ABCB2* and *TAP2/ABCB3* genes are half transporters that form the pump in the endoplasmic reticulum that complexes peptides with HLA class I molecules for antigen presentation on the cell surface. Rare mutations in each of these genes have been identified in patients with immune deficiency. An allele of the *TAP2/ABCB3* gene (M577V) has been identified that is present at 5% in the general population and is associated with the presence of autoantibodies in patients with Sjogren syndrome. This allele is also associated with the altered presentation of peptides on HLA class I

molecules. Tumor cells can potentially evade the immune system by failing to present class I antigens, and mutations in *TAP* genes have been found in cancer cell lines that are class I negative [21].

PSEUDOXANTHOMA ELASTICUM/ABCC6

Pseudoxanthoma elasticum is an autosoma recessive disease characterized by skin laxity and vision impairment characterized by angioid streaks and occlusion of blood vessels. Calcification of elastic fibers is a diagnostic feature. PXE is caused by mutations in the *ABCC6* gene. Interestingly, there is considerable variation in the presentation of PXE even within affected individuals in the same family, suggesting that the clinical manifestations of PXE are biochemically removed from the function. *ABCC6* is expressed predominantly in the liver and kidney and is proposed to transport a critical metabolite into or out of the blood. Mutation carriers of *ABCC6* variants have been associated with an increased risk of cardiovascular disease [21].

DILATED CARDIOMYOPATHY/ABCC9

Individuals with dilated cardiomyopathy present with heart failure and rhythm disturbances. ABCC9 displays low affinity binding to sulfonylurea and is a major regulator of ATP dependent potassium channels in muscle. ABCC9 mutations have been identified in two patients with dilated cardiomyopathy and these variants have been shown to disrupt catalytic K(ATP) channel gating [21].

MULTIDRUG RESISTANCE

Cells exposed to toxic compounds can develop resistance by a number of mechanisms including decreased uptake, increased detoxification, alteration of target proteins, or increased excretion. Several of these pathways can lead to multidrug resistance (MDR), in which the cell is resistant to several drugs in addition to the initial compound. This is a particular limitation to cancer chemotherapy and the MDR cell often displays other properties, such as genome instability and loss of checkpoint control, which complicate further therapy. ABC genes have an important role in MDR and several of them are associated with drug transport (Table 2).

The best characterized ABC drug pump is the *ABCB1* gene, formerly known as *MDR1* or *PGY1*. *ABCB1* was the first human ABC transporter cloned and characterized through its ability to confer a multidrug resistance phenotype to cancer cells that had developed resistance to chemotherapy drugs [51]. P-gp efficiently removes cytotoxic drugs and many commonly used pharmaceuticals from the lipid bilayer. Its broad substrate specificity presumably reflects a large, polymorphous drug-binding domain or domains within the transmembrane segments. ABCB1 has been demonstrated to be a promiscuous transporter of hydrophobic substrates, hydrophobic drugs including drugs including colchicine, VP16, adriamycin and vinblastine as well as lipids, steroids, xenobiotics, and peptides [52]. The gene is thought to have an important role in removing toxic metabolites from cells, but is also expressed in cells at the blood–brain barrier and presumably has a role in transporting compounds into the brain that cannot be

delivered by diffusion. ABCB1 also affects the pharmacology of the drugs that are substrates and a common polymorphism in the gene affects digoxin uptake [3]. Because P-gp binds many different hydrophobic compounds, it has been easy to find potent P-gp inhibitors. Two inhibitors that are used in the laboratory and in clinical trials that attempted to reverse drug resistance are the calcium channel blocker verapamil and the immunosuppressant cyclosporin A. As not all multidrug-resistant cells express P-gp, a search for other efflux pumps was initiated, leading to the discovery of the multidrug-resistance-associated protein 1 (MRP1, or ABCC1) [53].

Common Name	Systematic name	Tissue	Non-chemotherapy substrates	Chemotherapy substrates (known and suspected)	Defects in human disease	References
PGP/MDR1	ABCB1	Intestine, liver, kidney, placenta, blood–brain barrier	Neutral and cationic organic compounds, many commonly used drugs	Doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, homoharringtonine (STI-571)	None known; altered sensitivity to drugs	8
MDR2	ABCB4	Liver	Phosphatidylcholine, some hydrophobic drugs	Paclitaxel, vinblastine	Progressive familial intrahepatic cholestasis	31,33,66,67
MRP1	ABCC1	All tissues	Glutathione and other conjugates, organic anions, leukotriene C4	Doxorubicin, epirubicin, etoposide, vincristine, methotrexate	None known	20–24
MRP2, cMOAT	ABCC2	Liver, kidney, intestine	Similar to MRP1, non-bile salt organic anions	Methotrexate, etoposide, doxorubicin, cisplatin, vincristine, mitoxantrone	Dubin–Johnson syndrome	24,60–63
MRP3	ABCC3	Pancreas, kidney, intestine, liver, adrenal glands	Glucuronate and glutathione conjugates, bile acids	Etoposide, teniposide, methotrexate, cisplatin, vincristine, doxorubicin	None known	37,38
MRP4	ABCC4	Prostate, testis, ovary, intestine, pancreas, lung	Nucleotide analogues, organic anions	Methotrexate, thiopurines	None known	39,40
MRP5	ABCC5	Most tissues	Nucleotide analogues, cyclic nucleotides, organic anions	6-Mercaptopurine 6-Thioguanine	None known	41,42
MRP6	ABCC6	Liver, kidney	Anionic cyclic pentapeptide	Unknown	Pseudoxanthoma elasticum (substrate unknown)	43–46,58
MXR, BCRP, ABC-P	ABCG2	Placenta, intestine, breast, liver	Prazosin	Doxorubicin, daunorubicin, mitoxantrone, topotecan, SN-38	None known	25–29,54
BSEP, SPGP	ABCB11	Liver	Bile salts	Paclitaxel	Progressive familial intrahepatic cholestasis	30,47,48, 64,65
ABCA2	ABCA2	Brain, monocytes	Steroid derivatives, lipids	Estramustine	Intracellular steroid transport	7,34,35

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Table 2. Tissue localization and possible functions of ABC transporters

MRP1 is similar to P-gp in structure, with the exception of an aminoterminal extension that contains five-membranespanning domains attached to a P-gp-like core. MRP1 recognizes neutral and anionic hydrophobic natural products, and transports glutathione and other conjugates of these drugs, or, in some cases — such as for vincristine — co-transports unconjugated glutathione [54, 55]. The discovery of MRP1 stimulated a genomic search for homologues, leading to the discovery of eight additional members of the ABCC subfamily of transporters, of which six have been studied in some detail. Like MRP1, some of these MRPs have the five transmembrane amino-terminal extension (ABCC2, ABCC3 and ABCC6, also named MRP2, 3, and 6), whereas others do not. Many MRP family members transport drugs in model systems and therefore have the potential to confer drug resistance [56]. Some anticancer drugs, such as mitoxantrone, are poor substrates for MDR1 and MRP1. Selection for mitoxantrone resistance results in multidrug-resistant cells that produce a more distant member of the ABC transporter family, ABCG2 — also known as

MXR (mitoxantrone-resistance gene), BCRP (breast cancer resistance protein) or ABC-P (ABC transporter in placenta). This transporter is thought to be a homodimer of two half-transporters, each containing an ATP-binding domain at the amino-terminal end of the molecule and six transmembrane segments [57].
The first two original *ABCG2* genes that were cloned from resistant cells encoded proteins with either a threonine or glycine substituted for arginine at amino acid 482, giving them much broader substrate specificity, including the ability to transport doxorubicin. This finding, together with many well-documented mutations in *P-GP*, shows that even single amino-acid substitutions can change substrate specificity. Other ABC family members have been associated with drug resistance. For example, the bile salt export protein (BSEP, also known as ABCB11), first reported as the 'sister of P-gp' (SPGP), is expressed at high levels in liver cells, and in transfection experiments it confers low level resistance to paclitaxel [58]. MDR3 (sometimes called MDR2), a phosphatidylcholine flippase that is closely related to P-gp, normally transports phospholipids into bile, but can transport paclitaxel and vinblastine, albeit inefficiently unless it is mutated [57]. Finally, *ABCA2* is overexpressed in estramustine-resistant cells [59]. Estramustine is a nitrogen mustard derivative of oestradiol, so *ABCA2* — which is expressed intracellularly in endosomal/lysosomal vesicles — might participate in steroid transport. Although the lung resistance protein (LRP) is not an ABC transporter, it is frequently included in discussions of drug resistance, as it is expressed at high levels in drug-resistant cell lines and some tumours [57]. LRP is a major vault protein found in the cytoplasm and on the nuclear membrane. Vaults are large ribonucleoprotein particles that are present in all eukaryotic cells. Their shape is reminiscent of the nucleopore central plug, and the major vault proteins account for 70% of their mass. Although their role in normal physiology is not yet established, vaults might confer drug resistance by redistributing drugs away from intracellular targets [57].

ABC TRANSPORTERS IN HUMAN CANCERS

Although it seems likely that cancer cells use several different types of ABC transporter to gain drug resistance, most clinical studies have focused on P-gp. Early studies showed that P-gp was highly expressed in colon, kidney, adrenocortical and hepatocellular cancers. Initially, there was hope that increases in P-gp expression alone could explain cancer drug resistance. However, the failure of these cancers to respond to drugs that are not P-gp substrates indicated that other factors are involved, and attention turned to cancers that acquire resistance following chemotherapy. In seeking to define the role of P-gp in drug resistance, researchers have assumed that P-gp expression is highest in tumours that are dependent on expression for survival, that expression impairs response to chemotherapy, and that expression levels increase as tumours become drug resistant. On the basis of these assumptions, clinical trials aimed at increasing chemotherapy sensitivity in drug-resistant tumours, through inhibition of P-gp, have been implemented. So, does *P-GP* expression confer drug resistance in human cancer? Most studies that correlate *P-GP* expression with clinical outcome have been small, retrospective, single-institution studies with insufficient power to draw reliable conclusions. One problem with designing a study that provides statistically valid results is that methods for detecting P-gp expression are imperfect. This is primarily due to the lack of specificity
of many commonly used anti-P-gp antibodies. To complicate matters, researchers also use different methods to quantify expression, to control for tumour

heterogeneity, and to account for the presence of normal tissue in tumour biopsies. Despite efforts to bring uniformity to P-gp quantification, it is still difficult to discern valid from invalid data.
Expression of *MRP1* has also been analysed in clinical samples. Antibodies against MRP1 seem to be more specific than those that recognize P-gp, and MRP1 is highly expressed in leukaemias, oesophageal carcinoma and non-small-cell lung cancers. Conclusions about expression levels of other ABC transporters in human tissue await more extensive analysis [57].

LEUKEMIA

The most uniform associations between MDR1/P-gp expression and drug resistance have been reported in acute myelogenous leukaemia (AML). P-gp expression has been reported in leukaemic cells from about one-third of patients with AML at the time of diagnosis, and more than 50% of patients at relapse; higher levels occur in certain subtypes, including secondary leukaemias. P-gp expression is correlated with a reduced complete remission rate, and a higher incidence of refractory disease — a surprising finding, as treatment regimens include not only the P-gp substrate daunorubicin, but also AraC, which is not a P-gp substrate [57]. Recent studies report that *P-GP* expression is associated with a poorer prognosis, although the magnitude of the effects on response and survival is probably not as great as initially thought. These clinical results are supported by *ex vivo* studies of leukaemic cells, which have shown that *P-GP* expression reduces the intracellular accumulation of daunorubicin. In addition, administration of a P-gp inhibitor increases daunorubicin accumulation in leukaemic cells [57]. *MRP1* and *LRP* expression have also been evaluated in leukaemia. Increased *MRP1* expression has been reported in chronic lymphocytic and prolymphocytic leukaemia cells. Expression levels are less frequently elevated in AML cells (10–34%) [60, 61], and these studies lead to different conclusions about whether MRP1 confers a poor prognosis. So far, the largest trial in untreated patients found no correlation between *MRP1* or *LRP* expression and prognosis, but observed a correlation between *PGP* expression and prognosis [57]. Finally, low expression levels of BCRP/MXR have been observed in AML cells [62]. Taken together, the clinical data support a role for PGP in drug resistance in AML patients, and for MRP1 expression in chronic lymphocytic and prolymphocytic leukaemias. Additional studies are needed to confirm and extend these findings [57].

BREAST CANCER

Detection of transporters in solid tumour samples has been more difficult. A 1997 meta-analysis of 31 reports from 1989–1996 found that 41% of breast tumours expressed *P-GP*. *P-GP* expression increased after therapy and was associated with a greater likelihood of treatment failure. However, there was considerable interstudy variability, preventing a solid conclusion about the role of *P-GP* in breast cancer. Recent imaging studies using 99mTc (technetium)-sestamibi (Cardiolite), a transport substrate recognized by *P-GP*, indicate that its activity is increased in breast carcinomas [63]. Whether the *MRP1* expression levels associated with breast cancer are enough to confer drug resistance is not yet resolved. As *MRP1* is expressed ubiquitously, it is not surprising that using reverse transcriptase polymerase chain reaction (RT—PCR), *MRP1* mRNA can be detected in all breast cancer samples at levels comparable to that in normal tissues. One immunohistochemical analysis of a

series of resected invasive primary breast carcinomas reported a correlation between relapse-free survival and *MRP1* expression [57].

NEUROBLASTOMA

NB is the second most common solid cancer in young children accounting for 9% of all childhood cancers [64]. It is characterised by a heterogeneous clinical behaviour that ranges from spontaneous regression in 10% of all cases to rapid and largely treatment resistant progression with fatal outcome. At present, patient risk classification includes *N-MYC* gene amplification, 1p chromosomal deletion, tumour stage, and age of the child at diagnosis [65, 66]. Amplification or overexpression of the *N-MYC* oncogene is present in about 25–30% of primary untreated NBs, which is associated with advanced stage disease, rapid progression and unfavourable prognosis [67]. Moreover, this patient subgroup often demonstrates a multiple drug resistant (MDR) phenotype that develops from exposure to chemotherapeutic agents and increases with intensity of the therapy accommodated. NB treatment includes induction chemotherapy, maintenance of high dose chemotherapy, radiotherapy, tumour surgery and consolidation therapy. Chemotherapeutic protocols combine alkylating agents with microtubule active drugs, topoisomerase inhibitors and antibiotics. Anti-neoplastic drugs are effectively used in the treatment of NB patients. Following initial treatment with cytotoxic drugs, NB tumours are highly chemoresponsive, displaying significant partial or complete remission in about 80% of tumours, even those with unfavourable prognostic outcome [68]. Although many high-risk NBs initially respond to the first cycles of intensive chemotherapy, they frequently become refractory to treatment as the disease progresses. *N-MYC* clearly contributes to the drug-resistant phenotype of NB, as amplification of this oncogene is strongly associated with rapid tumor progression and poor prognosis [69, 70]. The role of *MDR1* gene in mediating multidrug resistance in neuroblastoma is still unclear. Chan and associates [71] demonstrated that *P-GP* expression in neuroblastoma independently predicted for poor outcome, but other studies of *MDR1* expression in this malignancy have failed to confirm these results [72]. Controversy regarding the contribution of MDR1 to the chemoresistant phenotype was heightened by a study, which reported that *P-GP* expression in neuroblastoma was restricted to the normal infiltrating stroma cells [73]. In neuroblastoma cell lines, induced to differentiate by exposure with retinoic acid , *MDR1* expression increases in parallel with other markers of neuronal differentiation [74] and this increased expression was not associated with the expected decrease in accumulation of cytotoxic drugs. As result of these disparate data, the contribution of the *MDR1* gene to either drug resistance or to patient outcome in neuroblastoma remains ill-defined. *MDR1* gene expression failed to predict for outcome in this tumour, but *MRP1* gene expression is a powerful prognostic indicator for children with neuroblastoma [72]. High *MRP1* expression but low *MDR1* expression was observed in tumours with *N-MYC* amplification and the expression of these two genes was not correlated in this subset of tumours [73]. High levels of *MRP1* expression were strongly associated with reductions in both survival and event-free survival. Altough *MRP1* is a direct target of N-Myc [75], a multivariate analysis demonstrates that *N-MYC* amplification had no prognostic value when *MRP1* expression was included as a prognostic factor, may be N-Myc governs the transcription of critical genes, such as *MRP1*, conferring multidrug resistance [72]. Although available evidence strongly suggests that MRP1 is critically associated with the drug-resistant behavior of primary NB [72], this drug efflux pump does not appear to mediate resistance to either

alkylating agents or platinum compounds [57]. Members of both these classes of compounds, such as cisplatin and cyclophosphamide, are commonly used in the treatment of NB. Thus, despite the high levels of *MRP1* observed in many aggressive NBs at diagnosis, the use of these non-MRP1 substrate drugs may explain why the majority of NB do initially respond to chemotherapy. Nevertheless, over half of these previously responsive tumors will eventually relapse with chemoresistant disease, suggesting the development of additional drug-resistance mechanisms [75]. Also *MRP4* is expressed in primary neuroblastoma and also its overexpression is significantly associated with *N-MYC* amplification and *MRP1* expression. The drug resistance phenotype of MRP4 has to date been thought to encompass primarily nucleoside analogues (including antiretroviral agents) and methotrexate. MRP4 is also able to confer significant resistance in vitro to the topoisomerase I poison irinotecan and its active metabolite SN-38. This drug is approved for treatment of colon and lung cancers and shows promising activity against a number of other cancers including cervical, ovarian carcinomas, and also neuroblastoma. Like *MRP1*, also high *MRP4* expression correlate with poor clinical outcome in neuroblastoma [44].

OTHER SOLID TUMOURS

Variability in expression is also a problem for other solid tumours. In ovarian cancer samples, 16–47% were found to express *P-GP*, as measured by immunohistochemistry. Critical analysis of these data reveals that *P-GP* is expressed by only about 20% of ovarian cancers when samples were taken at diagnosis. This makes it difficult to demonstrate a correlation between expression and outcomes, such as disease- free survival, particularly given the importance of cisplatin in therapy. In lung cancer samples, *MDR1* mRNA expression was reported to be increased in 15–50% of tumours. The incidence of *MRP1* expression is much higher (about 80%) in small-cell lung cancer (SCLC) samples. *MRP1* expression was detected in 100% of non-small cell lung cancers (NSCLC),with higher levels noted in 30% of the samples — this might not be surprising, given its ubiquitous expression in normal lung tissue. Immunohistochemical studies confirmed the predominantly plasma-membrane localization pattern of MRP1. Given the low levels of *MDR1* expression and the nearly ubiquitous expression of *MRP1*, lung cancer should be an excellent model in which to evaluate the role of MRP1-specific inhibitors. Sarcomas represent another malignancy in which P-gp expression seems to be important for drug resistance. Immunohistochemical studies of both soft-tissue sarcomas and osteosarcomas revealed a strong association between P-gp expression, relapse-free survival and overall survival. Other methodologies, however, have been used to substantiate and refute these findings, and there has been no consensus regarding the effect of P-gp on survival in sarcomas [57].

DEVELOPMENT OF DRUG RESISTANCE ABC-MEDIATED

Three mechanisms have been proposed to understand how an alterated expression of ABC transporters can result in drug resistance in human tumours (Fig. 4). According to intrinsic resistance model, tumor cells are intrinsically resistant due to high constitutive expression of ABC transporters. This high expression may be a reflection of the expression levels in the tissue of origin (for example, colon) or a result of a change in the expression of a tumor suppressor gene (e.g. p53) during progression to

the malignant state that influences ABC genes transcription. In this scenario, chemotherapy has little effect on the intrinsically resistant tumor. Acquired resistance model is the result of inherent genomic instability, thus an initially sensitive tumor acquires a subpopulation of mutant cells that constitutively express high levels of ABC transporters. In this scenario, chemotherapy eradicates drug-sensitive cells, but the resistant subset of cells survives treatment and may ultimately replicate.

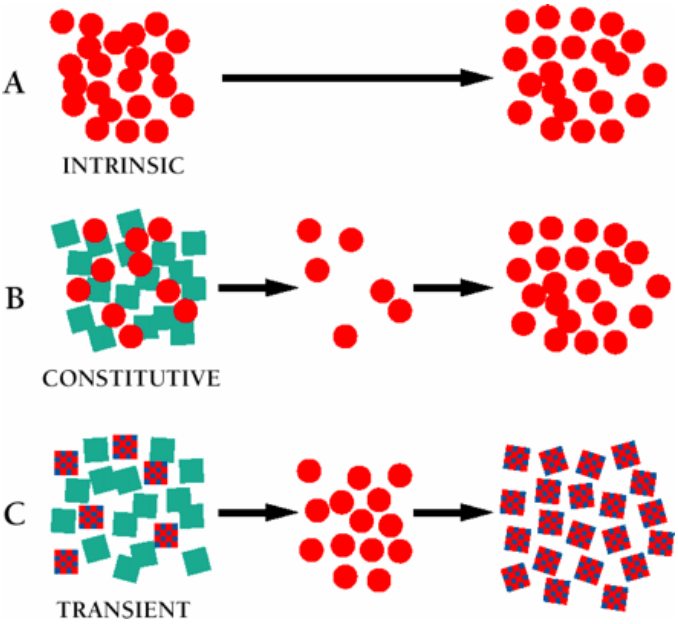


Figure 4. Models of drug resistance in tumour cells. Three proposed mechanisms (intrinsic, acquired and inducible) by which overexpression of *MDR1* can result in drug resistant in human tumours.

Finally, the inducible resistance model propose that a subset of tumor cells within a drug-sensitive population are able to rapidly (and transiently?) induce ABC genes expression in response to stress stimuli. Upon exposure to chemotherapy, sensitive cells are killed, but the inducible subset increases the expression of ABC genes, permitting survival and repopulation. Whether/how long ABC transporters levels remain elevated in surviving tumor cells in the clinical setting has not yet been investigated [76].

ROLE OF TUMOUR STEM CELLS IN THE DEVELOPMENT OF DRUG RESISTANCE

The discovery of cancer stem cells in solid tumours has changed our view of carcinogenesis and chemotherapy. One of the unique features of the bone marrow stem cells that are required for normal haematopoiesis is their capacity for self-renewal. In the haematopoietic system, there are three different population of multipotent progenitors-stem cells with high capacity for long-term renewal, stem cells with high capacity for short-term renewal, and multipotent progenitors that cannot renew but differentiate into the varied lineages in the bone marrow [77]. The multipotent progenitors and their derived lineages undergo rapid cell division,

allowing them to populate the marrow. The factors that determine the self-renewing capacity of a cell, and how cancer cells acquire this ability , are not yet understood. Pluripotent stem cells that possess both self-renewal capabilities and the ability to generate an organ-specific, differentiated repertoire of cells exist in organs other than the haematopoietic system and these can be studied to gain better insight into the stem-cell biology of a tumour. The concept of organ stem cells is difficult when one considers the many different cell types and functions of an organ, but emerging evidence indicates such pluripotent stem cells exist. In the normal mammary gland, for example, three cell lineages have been described — myoepithelial cells that form a basal cell layer, ductal epithelial cells, and milk-producing alveolar cells. Although transplantation studies in mice have demonstrated that most mammary cells have a limited capacity for self-renewal, clonal populations that can recapitulate the entire functional repertoire of the gland have been identified. In an elegant study, human mammary epithelial cells derived from reduction mammoplasties were used to generate non-adherent spheroids (designated mammospheres) in cell culture and demonstrate the presence of the three mammary cell lineages. More importantly, the cells in the mammospheres were clonally derived, providing evidence for a single pluripotent stem cell [78]. These same approaches are being used to isolate and characterize breast cancer stem cells. In the haematopoietic system as well as in other normal tissues, the normal stem cell must be both self-renewing and pluripotent. Although stem cells can self-renew, they are generally quiescent, spending most of their time in G0. Because stem cells can repair their DNA as they self-renew, they have the potential to accumulate mutations acquired after exposure to carcinogens. If tumours arise from stem cells, the accumulation of these mutations might be what we have come to recognize as the ‘multistep process of carcinogenesis’. So do cancer stem cells arise from normal stem cells, or do they arise from differentiated cells that acquire self-renewal capacity, or both? Does the innate resistance of normal stem cells to radiation and toxins contribute to the failure of some cancer therapies? How can we exploit our knowledge of stem-cell biology to specifically target these cells and improve therapy?

CANCER STEM CELLS

Cells with stem-cell qualities have been identified in malignancies of haematopoietic origin and in some solid tumours. The existence of such a population would imply that the stem cell represents the cell of origin for the tumour, as illustrated in Fig. 5 [79]. One can predict that such cancer stem cells represent only a small fraction of a tumour, as they possess the capability to regenerate a tumour, and most cancer cells lack this regenerative capability. Studies of acute myelogenous leukaemia have shown that only 0.1–1% of all cells have leukaemia-initiating activity [80]. These leukaemia-initiating cells have many markers and properties of normal haematopoietic stem cells [81, 82]. So it is believed that leukaemia arises from a stem cell that becomes transformed and gives rise to a large population of clones that proliferate but cannot self-renew or fully differentiate. Similar populations of self renewing cells, such as those that carry the chromosomal translocation t(9;22)(q34;q11), which forms the *BCR–ABL* fusion gene, have also been identified in patients with chronic lymphocytic leukaemia and chronic myelogenous leukaemia (CML) [83]. Evidence for the existence of a pluripotent cell in solid tumours includes clinical observations with human teratocarcinomas, an experiment of nature in which differentiated tissues such as muscle and bone can appear in the tumour mass, and

from the observation that mouse teratocarcinoma cells can produce a normal mouse. Instead of haematopoietic markers, stem cells identified from solid tumours usually express organ-specific markers. In eight of nine human breast cancer samples, for example, a tumorigenic stem-cell population was found that expressed the unique cell surface marker profile CD44+CD24–/low Lin– [78] This population was enriched 50- to 100-fold with cells able to form tumours in mice. The resulting tumours possess the phenotypic heterogeneity found in the original tumour population, including both tumorigenic and non-tumorigenic cells. In another study, overexpression of the WNT family of genes, important regulators of normal cell development, led to expansion of the mammary-stem-cell pool and cancer susceptibility. Finally, stem cells with a capacity to self-renew and undergo pluripotential differentiation have been isolated from human central-nervous-system tumours [84, 85]. These cells were reported to express CD133, a cell surface antigen known originally as a marker of haematopoietic stem cells and later observed as a marker of stem cells in other normal tissues [79].

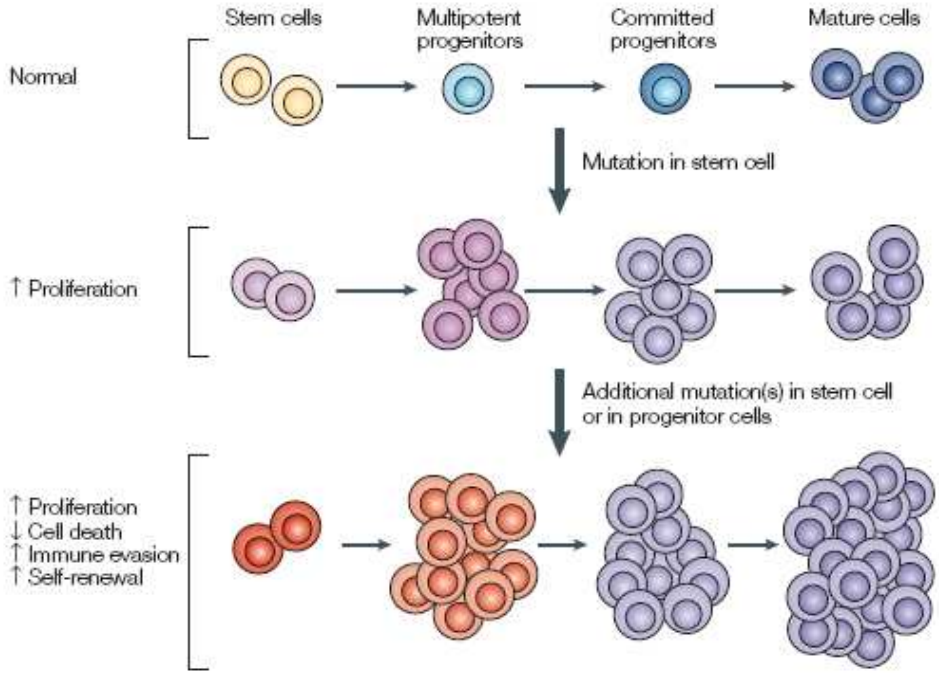


Figure 5. Normal stem cells give rise to multipotent progenitor cells, committedprogenitors and mature, differentiated cells. Mutations in a stem cell give rise to a stem cell with aberrant proliferation and result in a pre-malignant lesion. Additional mutations lead to the acquisition of further increased proliferation, decreased apoptosis, evasion of the immune system, and further expansion of the stem-cell compartment that is typical of malignant tumours.

The exact origin of pluripotent stem cells in tumours might vary. They could arise from the malignant transformation of a normal stem cell that has accumulated oncogenic insults over time. Alternatively, the original tumour cell could be a more differentiated cell that develops the capacity for continual self-renewal, thus acquiring the properties of a stem cell. Distinguishing between these two might be difficult. Evidence that cells other than stem cells can acquire the ability to undergo self-renewal has been recently provided in studies examining the progression of CML [83]. The chronic phase of the disease occurs when a stem cell acquires the expression of

the BCR–ABL fusion protein, leading to increased proliferation of cells within the granulocyte–macrophage progenitor pool and their downstream progeny. It is hypothesized that progression to blast crisis follows additional genetic or epigenetic events that confer progenitor cells with the capacity to self-renew, making them indistinguishable from a leukaemic stem cell. Further proof is needed to confirm that progression to blast crisis occurs at the level of the progenitor pool, but the proposal that the stem-cell compartment is not rigidly defined is attractive and suggests a degree of plasticity in cancer. Cancer stem cells (with either inherent or acquired capabilities for self-renewal) give rise to cells that lack long-term self-renewal capability but retain a finite ability to divide. In normal physiology, this would be called 'differentiation', as the cell acquires traits specific to its place in the tissue. But in cancer, cells lack the ability to undergo differentiation into phenotypically mature cells. A limited amount of differentiation often does occur, giving rise to the well-known histopathological and molecular distinctions between tumours. In fact, the further along this pathway the cancer cell travels, the more differentiated and the more like its normal counterpart it becomes, accordingly demonstrating a slower growth rate. Where the so-called 'de-differentiated' tumours fit along this continuum is uncertain, but it is possible that self-renewal might be a property that represents a higher order of differentiation. Therefore, the cancer stem cell shares many properties of the normal stem cell. It is generally accepted that normal stem cells show properties that provide for a long lifespan such as relative quiescence, resistance to drugs and toxins through the expression of several ATP binding cassette (ABC) transporters, an active DNA repair capacity, and a resistance to apoptosis. It follows that cancer stem cells might also possess these resistance mechanisms. The paradigm that drug resistance originates in the stem-cell phenotype might stimulate new strategies for the development of anticancer therapies [79].

DRUG TRANSPORTERS IN STEM CELLS

Stem cells have many properties that separate them from mature, differentiated cells. In addition to their ability to self-renew and differentiate, they are quiescent, dividing infrequently. They also require specific environments comprising other cells, stroma and growth factors for their survival. One particularly intriguing property of stem cells is that they express high levels of specific ABC drug transporters. For example, haematopoietic stem cells express high levels of *ABCG2*, but the gene is turned off in most committed progenitor and mature blood cells [86]. The two ABC transporter-encoding genes that have been studied most extensively in stem cells are *ABCB1*, which encodes P-glycoprotein [57], and *ABCG2*[86, 87]. Along with *ABCC1*, they represent the three principal multidrug-resistance genes that have been identified in tumour cells. These genes, members of the ABC-transporter superfamily, are promiscuous transporters of both hydrophobic and hydrophilic compounds [3, 57].These transporters also have important roles in normal physiology in the transport of drugs across the placenta and the intestine (more accurately, the retention of drugs in the intestinal lumen), and are important components of the blood–brain and blood–testis barriers. By using the energy of ATP hydrolysis, these transporters actively efflux drugs from cells, serving to protect them from cytotoxic agents. Mice deficient in either *Abcg2*, *Abcb1* or *Abcc1* are viable, fertile and have normal stem-cell compartments. This indicates that none of these genes are required for stem-cell growth or maintenance. However, these knockout mice are more sensitive to the effects of drugs such as vinblastine, ivermectin, topotecan and mitoxantrone, consistent with a role for these

ABC transporters in protecting cells from toxins. The drug-transporting property of stem cells conferred by these ABC transporters is an important marker in the isolation and analysis of haematopoietic stem cells. Most cells accumulate the fluorescent dyes Hoechst 33342 and rhodamine 123, but stem cells do not, as these compounds are effluxed by *ABCG2* and *ABCB1*, respectively. Because they don't accumulate these fluorescent dyes, stem cells can be sorted by collecting cells that contain only a low level of Hoechst 33342 fluorescence [79]. These cells are referred to as 'dull cells' or 'side population' (SP) cells. The term side population was coined because during flow-cytometry analysis, SP cells are visualized as a negatively stained 'side population' to one side of the majority of cells on a density dot plot. A large fraction of haematopoietic stem cells are found in the SP fraction and when isolated from mice and transplanted into irradiated mice, small numbers of these SP cells can reconstitute the bone marrow, demonstrating that these cells are pluripotent. SP cells can be isolated from many tissues including the brain, breast, lung, heart, pancreas, testes, skin and liver, and these cells might represent lineage-specific stem cells [88].Hoechst-33342 staining of bone marrow from *ABCG2*-null mice fails to detect SP cells. However, the lack of staining for SP cells occurs not because these cells are absent, but because the lack of *ABCG2* expression allows these cells to accumulate Hoechst dye and become fluorescent [79].

SP CELLS IN TUMOURS AND CELL LINES

Once it was recognized that stem cells were predominantly found in the SP fraction, it became possible to sort and purify stem cells from virtually any population of cells or tissue. SP cells were identified in 15 of 23 neuroblastoma samples and in neuroblastoma, breast cancer, lung cancer and glioblastoma cell lines [89]. Furthermore, analysis of several cell lines that had been maintained in culture for long periods of time demonstrated a small population of SP cells. In the rat glioma C6 cell line, a population of SP cells was separated from a population of non-SP cells. Through the use of growth factors, investigators maintained these cells in culture, and showed that only the SP cells gave rise to both populations and produced cells with both neuronal and glial markers that were tumorigenic in mice [90]. This latter study provided strong evidence that in this cell line the SP population reflected a population with a capacity for self-renewal and limited maturation. However, this isolation approach is imperfect as the SP compartment is composed of stem and non-stem cells, and some stem cells are not in the SP fraction. For example, non-stem-cell tumour cells often express *ABCG2* and *ABCB1*. These genes are highly expressed in drug-resistant cells, and histopathological studies have reported increased expression of the ABCB1 transporter in more differentiated tumours [79]. In addition, in a range of cell lines, differentiating agents induce expression of *ABCB1*, inhibit cell growth, and increase the expression of markers of maturation [74]. Additional limitations exist in using cancer cell lines cultured *in vitro* to study stem-cell biology and drug resistance. Although SP cells and cells with stem-cell properties have been reported in cultured cell lines, it is difficult to reconcile the hypothesis that only a small fraction of cells in culture possess stem-cell characteristics with the rapid doubling time of cells in culture. Current paradigms envision a small stem-cell compartment possessing cells with the capacity for perpetual self-renewal existing alongside a much larger proliferative compartment with cells that have a finite ability to proliferate before presumably arresting and/or undergoing apoptosis. These paradigms can explain the low cloning efficiency of most cell lines, their inefficiency at colony formation in soft

agar, and their limited tumorigenicity. However, none of these models can explain how the stem cells remain a constant fraction of the total population, if indeed they do. Any proposal will require stem cells to divide slowly, and must recognize that in a cell line derived from a solid tumour the number of cells undergoing apoptosis is relatively small. One possibility is that there is an interchange of cells between a proliferative compartment and the stem-cell pool. That such an interchange might occur is not improbable, as the cell line almost certainly originated from a stem cell with a proliferative advantage [21].

ABC TRANSPORTERS AND NEURAL STEM CELLS

The anatomical location and lineage specificities of NSCs were only established when they were finally identified in the subependymal region and in the hippocampal dentate gyrus (DG), where they divide to generate progenitors that migrate along the rostral migratory stream to differentiate in the olfactory bulb or to integrate into the surrounding hippocampal neural circuitry, respectively [91]. Similar to HSCs, these nestin+ NSCs may be defined operationally as cells that can continuously self-renew and have the potential to generate intermediate and mature cells of both glial and neural lineages [92]. Furthermore, NSCs have also been reported to differentiate into hematopoietic cells [93]. Tumor-tropic NSCs have also been observed in peripheral malignancies apart from those primary brain malignancies. Reactive astrocytosis induced by inflammatory cytokines released by microglia in response to a pathological process is characterized by an increase in glial fibrillary acidic protein (GFAP), showing that GFAP is a marker in the differentiation of NSCs into astrocytes. In the brain, the proteins MRP1, MRP4 and MRP5 (ABCC family) were clearly localized, by confocal laser scanning microscopy, to the luminal side of brain capillary endothelial cells [94]. The MRP4 and MRP5 proteins were also detected in astrocytes of the subcortical white matter [94] . Notably, MRP5 protein was present in pyramidal neurons [94]. Another study has revealed that MRP1 and MRP5 are more abundant in various brain cells than the other family members though MRP3 and MRP4 could also be detected in astrocytes. MRP proteins may thus contribute to the resistance of the brain to several cytotoxic and antiviral drugs [91]. A recent real-time reverse transcription-polymerase chain reaction (RT-PCR) assay has been used to investigate the specific expression pattern of the ABC subfamily-A transporters in the brain and has shown that neurons express predominantly *ABCA1* and *ABCA3*; astrocytes express *ABCA1, ABCA2 and ABCA3*; microglia express ABCA1 and oligodendrocytes express *ABCA2* and *ABCA3* [95]. With its expression in liver and brain, ABCB1 – the prototype of the B subclass of transporters – and ABCA1 regulate the high-density lipoprotein levels in the plasma and cholesterol contents of several cell types in these organs. Most interestingly, the ABCB1 transporter also shows strong expression in neurons of the hippocampus formation, particularly in the granule cells of the DG. Volk *et al*. [96] have demonstrated neuronal upregulation of *ABCB1* expression in the CA3/CA4 region and hilus of the hippocampus formation 24 h after inducing a status epilepticus in rat brains. In general, however, ABCB1 is predominantly localized in the apical membrane of capillary endothelial cells which form the Blood Brain Barrier, and in epithelial cells of the blood-cerebrospinal fluid barrier, while other cell types in the brain show little or no expression under normal conditions [96].

The SP phenotype in mouse NSCs was probably due to the expression of *ABCB1*. Recent results show that NSCs from mouse forebrain are contained in a population

distinct from the SP [97]. Moreover, other research data show that the ABCA2 transporter is widely expressed in early neural progenitors developed *in vitro* from ES cells [98]. *ABCA2* expression in the adult mouse and rat brains seems to be region-dependent because it is limited to the oligodendrocytic lineage – unambiguously excluded from astrocytes– and to a subset of cortical GABAergic inter-neurons and pyramidal glutamatergic neurons where it could be localized to lysosomal-related organelles. ABCA2 has also been suggested to be a marker of neural progenitors as it is expressed in the subventricular zone of the lateral ventricle and the DG of the hippocampal formation, sites of continual neurogenesis in the adult brain, and in nestin+ cells differentiated *in vitro* from ES cells. It was only very recently that the distribution and functional properties of the transporters were studied in human neural stem/progenitor cells (hNSPCs). It was found that more than half of the hNSPCs within neurospheres expressed nestin, an NSPC marker. Furthermore, all nestin+ cells simultaneously expressed *ABCB1*. Moreover, when the hNSPCs were isolated by fluorescence activated cell sorting (FACS) using the ABCB1 antibody, there was an increase in nestin+ cells compared to cells separated by control IgG. Taken together, these results suggest that this ABC transporter may contribute to neural stem/progenitor cell expansion *in vitro*. Further study revealed that cultured hNSPCs expressed functional *ABCB1* as well as *ABCG2* at the cell surface, and that their expression was downregulated during differentiation of hNSPCs, similar to the downregulation of ABCG2 in HSCs at the stage of lineage commitment [99, 100]. It was observed that both ABC transporters were downregulated during hNSPC differentiation, together with nestin downregulation and GFAP upregulation. The downregulation of nestin and upregulation of GFAP are considered to be indicators of stem/progenitor differentiation or maturation, and therefore it was proposed that ABCB1 and ABCG2 may be markers of neural stem/progenitor cells, and may have a functional role in upholding the undifferentiated status of hNSPCs [99, 100]. Consistent with this idea, it was found that ABCG2 had a high substrate-stimulated ATP-hydrolysis activity in these cells, further suggesting that the ABCG2 transporter was functionally active in hNSPCs and may play a regulatory role in the maintenance of the undifferentiated state, possibly through modulating the uptake of small hydrophobic molecules involved in differentiation [100]. The biological activities of ABCB1 and ABCG2 in stem cells are part of the normal tissue regeneration mechanism, probably conferring protection of the small stem cell population from cell death and enabling preservation of homeostasis under extreme stress conditions. Interestingly, the correlation between nestin and *ABCB1* expression in hNSPCs, and inhibition of hNSCPs proliferation by an ABCB1 inhibitor (cyclosporine A) at a very low dose, suggested that the ABC transporter may contribute to neural stem/ progenitor cell expansion [99, 100]. While *ABCA2* shows higher expression in nestin+ mouse neural progenitors, at later developmental stages it undergoes a conspicuous downregulation, persisting only in limited subsets of differentiated neurons [98]. Based on these results and the observations that the ABC transporters are expressed at high levels in hNSPCs but are downregulated in differentiated hNSPCs, these genes could potentially function as putative NSC markers in a similar way as p21CIP/WAF or musashi-1. For instance, while a high expression of *ABCB1/ABCG2* reflects maintenance of proliferating NSCs in an undifferentiated state, low expression characterizes progenitors differentiating into neurons and astrocytes, which by themselves do not (or only at a very low level) express *ABCB1/ABCG2* at the end of differentiation (Fig. 6) [99, 100]. The published studies on NSCs support the hypothesis that ABCB1 (MDR1) might be more important than ABCG2 in controlling the specific phenotype of NSCs [98-100], in contrast to the HSCs where ABCG2 is involved

in the SP phenotype and considered as its molecular determinant. In short, ABC transporters have emerged as an important new field of investigation in the regulation of stem cell biology, and manipulation of this system may promote stem cell amplification via a common defence mechanism adopted by these cells through their high expression of ABC membrane transporters [91].

ABC TRANSPORTERS AND HEMATOPOIETIC STEM CELLS

The developmental programs that regulate gene expression profiles are tightly controlled and can lead to cancer if perturbed. An increasing number of genes are being characterized that can function as lineage-restricted transcription factors, directing hematopoietic cell differentiation. For example, GATA-1 is required for erythropoiesis, PU.1 for myelopoiesis, and Ikaros for lymphopoiesis, with crosstalk possible between factors modulating differentiation. The differential gene expression patterns that direct hematopoietic stem cell (HSC) self-renewal versus differentiation are less well characterized, but are of great interest [101]. The cells enriched for long-term repopulating activity have been shown to express the Sca-1 antigen, the c-kit receptor tyrosine kinase, but none of a cocktail of differentiation lineage markers . Isolation of stem cells based on the efflux of fluorescent dyes has also been an efficient method to further purify stem cells, and it has been demonstrated that Rhodamine123 (Rho123) retention is low in the most primitive hematopoietic cells. While the Rho-low fraction provides long-term reconstitution following injection into lethally irradiated mice, the Rho-high fraction provides only short-term repopulation [102]. Hoechst 33342 is another fluorescent dye used for isolation of stem cell fractions. In combination with Rho123 staining, Hoechst low Rho-low cells are highly enriched for stem cell activity [101].

The first identified candidate transporter for the efflux of fluorescent dyes in HSCs was MDR1/ ABCB1. The P-gp is highly expressed on CD34+ hematopoietic cells [103], suggesting that efflux pump activity could be responsible for the low retention of Rho123 in primitive subsets of cells. The *CD34* gene codes for a transmembrane cell surface phosphoprotein that has been generally accepted as being a stem cell marker based on engraftment following bone marrow transplantation into baboons, humans, and mice [101]. However, in recent years, this has become a point of controversy in the field. It was first shown that murine HSCs could be CD34 negative [104]. Soon after, Goodell *et al*. demonstrated that a highly enriched stem cell fraction termed side-population (SP) cells could be isolated following Hoechst 33342 staining [105].

Human SP cells expressed low to undetectable levels of CD34, and the CD34– 38– cells could acquire CD34 during culture [101]. ABC transporter activity is involved in the differential Hoechst staining, since the SP tail could be eliminated by treatment with verapamil a known inhibitor of P-gp function. Interestingly, other studies have also demonstrated that both mouse and human CD34+ cells can be derived from CD34– cells. Transplantation studies in fetal sheep [106] also showed repopulating activity in the CD34– population. The concept evolved that CD34 expression may be up-or down modulated and that HSCs may therefore be CD34+ or CD34–. Isolation of murine bone marrow with the cell surface marker phenotype of Sca1+/c-kit+/lin– (KLS) can be used to enrich for SP cells, thus showing significant overlap between the two populations.

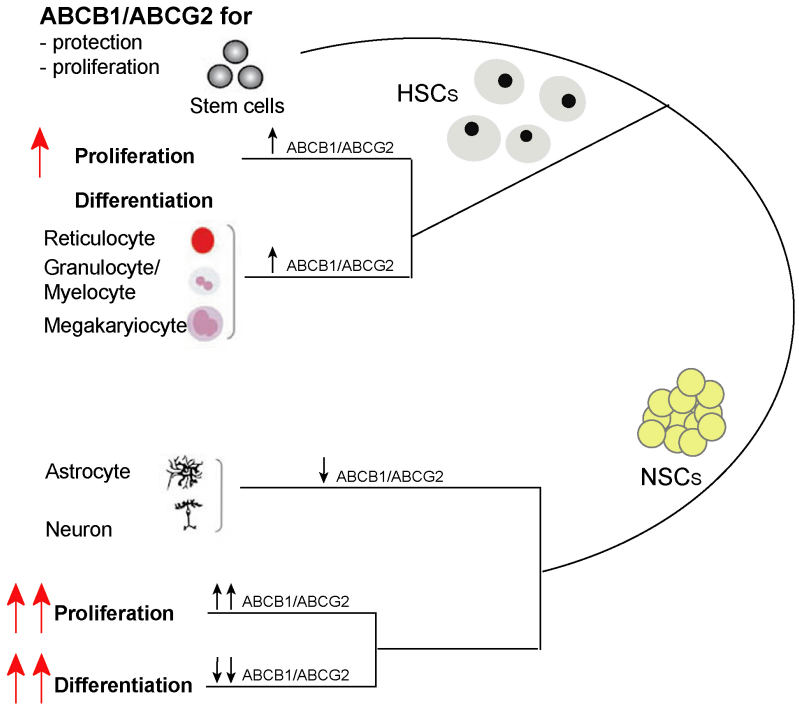


Figure 6. Schematic illustration of the effect of ABC transporters on stem cell proliferation and differentiation. (1) Expression of ABC transporters (especially ABCB1 and ABCG2) in stem cells like the NSCs, HSCs or pancreatic stem cells is thought to be essential for their *in vivo* proliferation and probably their self-renewal activity. (2) Enforced expression of ABCB1 or ABCG2 leads to enhanced proliferation in HSCs. (3) Downregulation of ABCB1 or ABCG2 in HSCs is observed with the differentiation of HSCs. (4) Downregulation of the ABC transporters in NSCs is observed with the differentiation into astrocytes or neurons. (5) The hypothesis suggests that expression of the ABC transporters in NSCs may have an effect on NSC differentiation or proliferation, such that significant upregulation of ABCB1 or ABCG2 expression may lead to an increase in self-renewal of NSCs, and correspondingly, a decrease in ABCB1 or ABCG2 expression may lead to increased differentiation of NSCs.

A common factor in all of these studies with Hoechst SP/CD34low/+ cells or KLS cells is that primitive hematopoietic cell populations capable of repopulating hosts express ABC transporter activity. It has now become clear that two specific transporters with unique substrate specificity are differentially expressed in this SP fraction during early hematopoietic differentiation. The phenotypes of knockout mice lacking expression of the two murine *mdr1a* and *mdr1b* homologs to the human MDR1 have been relatively mild, with each strain of mice showing normal health and viability [107, 108]. *mdr1a* and *mdr1b* knockout mice did show altered pharmacokinetics for a number of anticancer agents and hypersensitivity to the pesticide ivermectin. No significant hematologic defects were observed. However, numerous other ABC transporters have been identified in recent years that could serve compensatory endogenous functions. A growing family of MDR-associated proteins, as well as the half-transporters have also been described. A normal percentage of SP cells was recently described in the bone marrow from *mdr1a-1b–/–* mice, suggesting the presence of another transporter that has been previously uncharacterized regarding expression in primitive hematopoietic cells [88]. Further, inhibition of SP cell staining with either 2-deoxyglucose, a general inhibitor of ATP synthesis, or with verapamil

in the *mdr1a-1b–/–* background indicated that this activity was due to expression of an ABC transporter. This was not an entirely new concept, since P-gp expression studies in multidrug resistant myeloid leukaemia cells from patients showed dissociation of P-gp expression and dye efflux [60]. These initial studies strongly suggested that alternative transporters may be active in hematopoietic cells. The breast cancer resistance protein (BCRP), also called ABCG2 [30], was subsequently identified and characterized as a novel stem cell transporter [109]. ABCG2 has been most studied within hematopoietic cell populations. ABCG2 is the transporter responsible for the Hoechst 33342 dye efflux pattern in cells within the side population (SP) region and this population does not express MRP1-4, although MDR1a is expressed. Microarray analyses of cDNA from murine hematopoietic cell populations have identified *ABCG2* expression only within the most primitive subpopulations (C-Kit positive/Thy1 low/Sca1 positive/lineage negative) [110]. No other ABC transporter expression was identified within this fraction, consistent with the hypothesis that although there is considerable overlap of ABC transporter expression in various cell populations, *ABCG2* is relatively restricted to more primitive cells. Human stem cell products, including bone marrow, peripheral blood progenitor cells, and umbilical cord blood have also been evaluated for *ABCG2* expression. In human bone marrow, *ABCG2* mRNA expression was highest within the CD34+/CD38-/lineage negative/KDR+ population and also the SP+ population [86]. Umbilical cord blood and cytokine-mobilized peripheral blood progenitor cells also contain measurable ABCG2-positive populations, which are co-enriched with immunomagnetic selection for CD133+ and CD34+ [110]. It is currently unknown whether the recently described human CD133+/CD34- HSC expresses ABCG2. In addition to expressing *ABCG2*, the CD34+/lineage negative/SP+ hematopoietic cell population expresses aldehyde dehydrogenase which has been shown to protect hematopoietic stem cells from the active metabolites of cyclophosphamide. This suggests that primitive cell populations possess several different mechanisms for protection against environmental toxins, a feature that would necessarily complicate anticancer therapy, since cancerous cells typically arise from primitive cell populations and could take advantage of these protective mechanisms. Additional evidence suggests that ABCG2 may be expressed in a lymphoid progenitor cell population in cord blood. The lack of P-gp expression in a Hoescht-effluxing cell with a primitive immunophenotype is highly suggestive of *ABCG2* expression, although this was not directly tested. *ABCG2* expression has also been reported in early erythroid and natural killer cell populations, in both mice and humans [86, 88]. Little is known about the significance of *ABCG2* expression or dye efflux in these populations. The recent finding that protoporphyrin XI is an ABCG2 substrate suggests a possible physiologic role in early erythroid cell populations. Measurement of *ABCG2* mRNA expression by real-time RT-PCR has been studied as a potential tool for quantifying primitive HSC. One preliminary study showed a higher level of *ABCG2* mRNA expression in cord blood mononuclear cells compared to bone marrow and peripheral blood, and also a correlation of *ABCG2* mRNA levels with CD34+ and CD133+ cell frequency in mobilized PBSC [110]. Therefore, one interpretation is that *BCRP* expression may define primitive quiescent HSCs whereas MDR1 may be expressed in more “activated” repopulating HSC.

DRUG RESISTANCE IN CANCER STEM CELLS

Cancer cells can acquire resistance to chemotherapy by a range of mechanisms, including the mutation or overexpression of the drug target, inactivation of the drug,

or elimination of the drug from the cell. Typically, tumours that recur after an initial response to chemotherapy are resistant to multiple drugs (they are multidrug resistant). In the conventional view of drug resistance, one or several cells in the tumour population acquire genetic changes that confer drug resistance (Fig. 7a). These cells have a selective advantage that allows them to overtake the population of tumour cells following cancer chemotherapy. Based on the tumour stem-cell concept, an alternative model posits that the cancer stem cells are naturally resistant to chemotherapy through their quiescence, their capacity for DNA repair, and ABC-transporter expression (Fig. 7b).As a result, at least some of the tumour stem cells can survive chemotherapy and support regrowth of the tumour. In a third model of acquired resistance, drug-resistant variants of the tumour stem cell or its close descendants arise, producing a population of multidrug-resistant tumour cells that can be found in many patients who have recurrence of their cancer following chemotherapy (Fig. 7c).

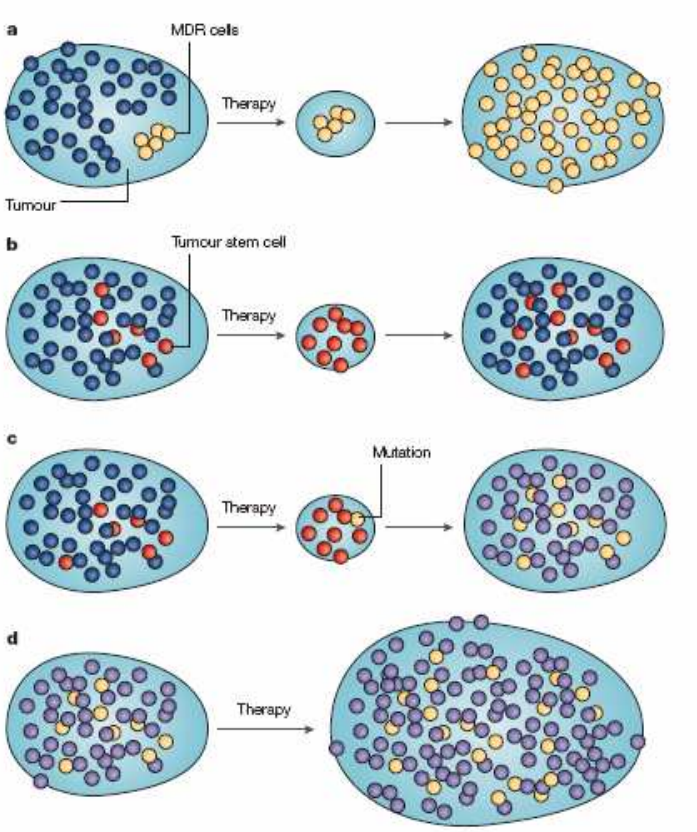


Figure 7. a) In the conventional model of tumour-cell drug resistance, rare cells with genetic alterations that confer multidrug resistance (MDR) form a drug resistant clone (yellow). Following chemotherapy, these cells survive and proliferate, forming a recurrent tumour that is composed of offspring of the drug-resistant clone. **b**) In the cancer-stemcell model, drug resistance can be mediated by stem cells. In this model, tumours contain a small population of tumour stem cells (red) and their differentiated offspring, which are committed to a particular lineage (blue). Following chemotherapy, the committed cells are killed, but the stem cells, which express drug transporters, survive. These cells repopulate the tumour, resulting in a heterogeneous tumour composed of stem cells and committed but variably differentiated offspring. **c)** In the 'acquired resistance' stem-cell model, the tumour stem cells (red), which express drug transporters, survive the therapy, whereas the committed but variably differentiated cells are killed. Mutation(s) in the surviving tumour stem cells (yellow) and their descendants (purple) can arise (by mechanisms such as point mutations, gene activation or gene amplification), conferring a drug-resistant phenotype. As in model **a**, the stem cell with the aquired mutations could be present in the population before therapy. **d)** In the 'intrinsic resistance' model, both the stem cells (yellow) and the variably differentiated cells (purple) are inherently drug resistant, so therapies have little or no effect, resulting in tumour growth.

The same mechanisms that allow stem cells to accumulate mutations over time, producing the long term consequences of exposure to irradiation or carcinogens, would then allow cancer stem cells to accumulate mutations that confer drug resistance to their abnormally developing offspring. As an example, genetic alterations such as those that upregulate *ABCB1* expression in human leukaemia and lymphoma cells could have originated in the stem cell [111]. In a final 'intrinsic resistance' model, both the stem cells and the variably differentiated cells are inherently drug resistant, so therapies have little or no effect, resulting in tumour growth (Fig. 7d).

An example of the latter is an intrinsically resistant cancer such as renal-cell cancer, in which *ABCB1* is expressed in all cells and contributes to chemotherapy tolerance. In this case, the resistance phenotype of the cancer stem cell persists in the committed, abnormally developing progenitors that comprise the proliferative pool of cancer cells. So in the cancer-stem-cell model of drug resistance, tumours have a built-in population of drug-resistant pluripotent cells that can survive chemotherapy and re-grow. Again, a parallel with normal stem cells can be found in stem-cell-driven recovery of normal tissues following chemotherapy. The rapid relapse observed with some tumours, at times within one cycle of chemotherapy, has a normal-tissue parallel in the repopulation of the bone marrow by normal haematopoietic stem cells and the recovery of the mucosa of the gastrointestinal tract, both of which usually occur within one 3-week cycle. Similarly, tumour recurrences that occur months to years after an original response to chemotherapy can be modelled on the slower recovery that is observed with hair follicles.

Although it is therapeutically attractive, the hypothesis that the intrinsic properties of stem cells alone provide the basis for drug resistance might be too simplistic. Recent studies of imatinib (Gleevec) resistance in patients with leukaemia provide an example of how ABC-transporter-mediated efflux in stem cells could facilitate, but not be solely responsible for, the acquisition of acquired mechanisms of drug resistance. Imatinib has been recently shown to be both a substrate and inhibitor of ABCG2, making it susceptible to efflux by a stem cell that expresses this ABC transporter [112-114]. The initial studies that reported imatinib-resistant leukaemia cells described 'acquired' mutations in the kinase domain of ABL in patients with CML or with acute lymphoblastic leukaemia associated with t (9;22)(q34;q11). These findings indicate that although the expression of drug transporters by the cancer stem cell might provide some level of drug resistance, an acquired mutation in ABL could confer higher levels of drug resistance. Although these mutations might have arisen during therapy, their existence before the administration of imatinib has not been excluded. Indeed, pre-existing mutations that confer resistance to imatinib have also been described in a subset of patients. These findings are reminiscent of the Goldie–Coldman hypothesis, proposed more than 20 years ago, that a small percentage of cells in a population harbouring intrinsic mutations confer drug resistance [115]. The Goldie–Coldman hypothesis would theorize that the cell acquiring the mutation is the stem cell. Although the expression of ABC transporters could render stem cells resistant to drugs, it is not the sole determinant of resistance, as the DNA-repair capacity of the cell and the reluctance to enter apoptosis could be equally or more important. Generally regarded as quiescent and non-dividing, stem cells would be expected to be inherently refractory to drugs that target either the cell cycle or rapidly dividing cells. To the extent that quiescence is an important mechanism of drug resistance in stem cells, agents will have to be developed that are effective in non-dividing cells. For example, studies with imatinib have shown that blocking BCR–

ABL-positive cells at the G1/S boundary *in vitro* had no significant impact on the ability of imatinib to induce apoptosis, indicating that imatinib is effective in non-dividing cells [21].

MECHANISMS AND STRATEGIES TO OVERCOME MULTIPLE DRUG RESISTANCE IN CANCER

By inhibiting the main transporters of chemotherapy drugs, it was thought that drug resistance could be avoided and tumour cells eliminated. Therefore, much effort has been devoted to the development of inhibitors of ABC transporters. There are many studies to overcome MDR by inhibiting MDR transporters, to suppress or circumvent MDR mechanisms. The use of anticancer drugs that could escape from the ABC transporters might be a solution to avoid drug resistance. Anticancer drugs which are not the substrates of ABC transporters are alkylating drugs (cyclophosphamide), antimetabolites (5-fluorouracil), and the anthracycline modified drugs (annamycin and doxorubicin-peptide). Another method to overcome resistance to anticancer drugs is to administer compounds that would not be toxic themselves, but would inhibit ABC transporters. The compounds that would reverse resistance against anticancer drugs are called MDR inhibitors, MDR modulators, MDR reversal agents or chemosensitizers. They may modulate more than one transporter. Clinical trials helped to unravel the problems associated with combination chemotherapy of anticancer drug(s) together with an MDR inhibitor. The first factor to be determined before embarking a clinical trial is to identify the ABC transporter protein involved in drug resistance and to utilize an anticancer drug that would benefit from inhibition of that transporter protein. The anticancer drug(s) utilized should match the transporter protein being inhibited. The second factor is to monitor the plasma concentrations and in vivo effectiveness of the tested MDR inhibitor in order to verify that an effective inhibitory concentration was in fact achieved in vivo. The pharmacokinetic interaction between the anticancer drug(s) and the MDR inhibitor must be searched and avoided to prevent a reduction in anticancer drug dosage [116].

FIRST-GENERATION MDR MODULATORS

Inhibiting P-gp and other ABC transporters has been extensively studied for more than two decades. Many agents of diverse structure and function that modulate MDR have been identified, including calcium channel blockers (e.g., verapamil), calmodulin antagonists, steroidal agents, protein kinase C inhibitors, immunosuppressive drugs (e.g., cyclosporine A), antibiotics (e.g., erythromycin), antimalarials (e.g., quinine), psychotropic phenothiazines and indole alkaloids (e.g., fluphenazine and reserpine), steroid hormones and anti-steroids (e.g., progesterone and tamoxifen), detergents (e.g., cremophorEL) and surfactants. First-generation MDR drugs had other pharmacological activities and were not specifically developed for inhibiting MDR. Their affinity was low for ABC transporters and necessitated the use of high doses, resulting in unacceptable high toxicity which limited their application. Clinical trials with first-generation MDR drugs failed for various reasons, often due to side effects. Many of the first-generation chemosensitizers were themselves substrates for ABC transporters and competed with the cytotoxic drugs for efflux by the MDR pumps. Therefore, high serum concentrations of the

chemosensitizers were needed to produce sufficient intracellular concentrations . These limitations prompted the development of new chemosensitizers that are more potent, less toxic and selective for the P-gp and other ABC transporters [116].

SECOND-GENERATION MDR MODULATORS

Second-generation chemosensitizers were designed to reduce the side effects of the first generation drugs. Second-generation MDR modulators have a better pharmacologic profile than the first generation compounds, still they retain some characteristics that limit their clinical usefulness. Co-administration of an MDR modulator usually elevate plasma concentrations of an anticancer drug by interfering its clearance or inhibiting its metabolism and excretion, thus leading to unacceptable toxicity that necessitates chemotherapy dose reductions in clinical trials down to pharmacologically ineffective levels. The affinity of second-generation MDR drugs towards ABC transporters was too low to produce significant inhibition of MDR in vivo at tolerable doses. Many of the anticancer drugs are substrates both for ABC transporter proteins and for the cytochrome P450 isoenzyme 3A4. Most of the second-generation MDR chemosensitizers are also substrates for cytochrome P450 3A4 and metabolized by this enzyme. The competition between anticancer agents and MDR modulators for cytochrome P450 3A4 activity may result in unpredictable pharmacokinetic interactions. Co-administration of a MDR drug may significantly elevate plasma concentrations of an anticancer drug by interfering with its clearance (e.g., via biliary elimination) or metabolism (e.g., via the cytochrome P450 system). This would increase the concentration of an anticancer drug leading to unacceptable side effects, necessitating dose reductions down to pharmacologically ineffective levels. However since the pharmacokinetic interactions between chemosensitizers and cytotoxic agents are unpredictable, reducing the dose of a cytotoxic agent may result in under- or over-dosing in patients. The unpredictable effects of second-generation MDR modulators on cytochrome P450 3A4-mediated drug metabolism limits the use of these second-generation modulators in the treatment of multidrug resistance. ABC transporters have well defined physiologic roles, often involving the elimination of xenobiotics, in regulating the permeability of the central nervous system (blood–brain barrier), the testes, and the placenta, thus preventing these systems from being exposed to cytotoxic agents circulating in the blood. Most of the second-generation MDR chemosensitizers are substrates for ABC transporter family. Inhibition of these transporters could lessen the ability of normal cells and tissues to protect themselves from cytotoxic agents. Inhibition of non target transporters may enhance adverse effects of anticancer drugs. Side effects due to modulation of MDR protein in normal tissues, especially blood–brain barrier should be monitored carefully to avoid neurological responses. Because of these problems, MDR inhibitors have not improved the therapeutic efficiency of anticancer drugs unless such agents lack significant pharmacokinetic interactions [116].

THIRD-GENERATION MDR MODULATORS

Third-generation molecules have been developed to overcome the limitations of the second generation MDR modulators. They are not metabolized by cytochrome P450 3A4 and they do not alter the plasma pharmacokinetics of anticancer drugs. Third-generation agents specifically and potently inhibit P-gp and do not inhibit other ABC transporters. None of the third-generation agents tested so far have caused clinically

relevant alterations in the pharmacokinetics of the co-administered anticancer drugs. Because of their specificity for P-gp transporters and lack of interaction with cytochrome P450 3A4, third-generation P-gp inhibitors offer significant improvements in chemotherapy without a need for chemotherapy dose reductions. Several such compounds are currently undergoing clinical trials in several cancer types. Schering AG has developed a quinolone derivative MDR modulator (MS-209). It is used in combination with the anticancer drug (doxetaxel) in advanced solid (breast and lung cancer) tumors. One of the most promising third-generation P-gp inhibitors is an anthranilamide derivative tariquidar (XR9576) which is developed by NCI/Xenova/QLT Company. In phase I and II studies with paclitaxel and vinorelbine in ovarian cancer, tariquidar gave successful results and phase III trials have been initiated with tariquidar in patients with non-small cell lung cancer. It binds specifically and non-competitively to the P-gp pump with a high affinity and potently inhibits the activity of the P-gp transporter. Tariquidar inhibits the ATPase activity of P-gp. Tariquidar is more potent and its inhibitory action on the P-gp transporter pump lasts longer in comparision to the effects of first- and second- generation P-gp modulators. In none of the clinical trials, tariquidar caused alterations in the pharmacokinetics of the coadministered cytotoxic agents such as paclitaxel, vinorelbine, or doxorubicin in patients with solid tumors. This allows the use of standard doses of these chemotherapeutic agents without the need for dose reduction. However, clinical trials with new third-generation agents are ongoing with the aim for a longer survival in cancer patients. This effort continues, but none of them has found a general clinical use so far [116].

NOVEL APPROACHES TO STRUGGLE WITH MDR MECHANISMS

The difficulties encountered with MDR inhibitors have led several alternative approaches to MDR therapy. These approaches can be divided in two groups. One group of studies consists of trials designed to inhibit MDR mechanisms in novel ways and the other group focuses on trials to circumvent MDR mechanisms. There are several approaches to inhibit mechanisms involved in regulation of MDR transporters. MDR protein gene expression in tumor cells is induced upon treatment with cytotoxic drugs, whereas this gene expression is inhibited by several pharmacological inhibitors that affect the signaling pathways. It was demonstrated that taxol stimulated MDR1 and cytochrome P450 3A4 (CYP3A4) gene expression via its direct interaction with and activation of the nuclear steroid and xenobiotic receptor (SXR) which led to increased drug resistance and faster drug clearance . Hence, antagonists of the nuclear steroid and xenobiotic receptor may be utilized in conjunction with anticancer drugs to cope with the induction of MDR1 and CYP3A4 [117]. Recent advances in antisense oligonucleotide technologies suggest an alternative and more specific way to cope with MDR than the use conventional MDR inhibitors [118]. Downregulation of ABC transporter proteins and enzymes involved in cancer cell resistance using antisense oligonucleotides may provide an efficient approach to overcome MDR.

Recent studies clarified the role of ceramide as a second messenger in cellular apoptotic signaling events. A decrease in ceramide production increases cellular resistance to apoptosis. It was demonstrated that glucosylceramide (GC), a simple glycosylated form of ceramide which results from elevated GlcCer synthase activity accumulates in multidrug resistant cancer cells and tumors derived from patients who are less responsive to chemotherapy [119, 120]. Overexpression of recombinant Glc-Cer synthase (GCS) confers resistance to adriamycin and to ceramide in GlcCer synthase-

transfected human breast cancer cells, suggesting that drug resistance is related to stimulation of glucosylation of ceramide and the resultant inhibition of drug induced apoptotic signaling [121]. Blocking the glycosylation of ceramide has been shown to increase cancer cell sensitivity to cytotoxics [122-124]. Drug combinations that enhance ceramide generation and limit glycosylation have been shown to enhance effectiveness of chemotherapy by inducing apoptosis in cancer cell models [123, 124]. The role of GlcCer synthase in drug resistance was demonstrated directly by antisense suppression of GlcCer synthase expression in MDR cells [125]. Downregulation of ceramide glycosylation using GCS antisense in adriamycin-resistant breast cancer cells restored cell sensitivity to adriamycin [125]. In another study, a novel amino-ceramide analog was shown to inhibit GlcCer synthase and thereby elevate ceramide production in MDR cells, enhancing drug-induced apoptosis [117]. These findings assign biological significance to ceramide metabolism and provides a promising approach to struggle with drug resistance. These results indicate that GlcCer synthase contributes to drug resistance in MDR cells by attenuating drug-induced formation of apoptotic ceramide and suggest that GlcCer synthase may represent a novel drug target in cancer MDR.

Living cells needs MDR mechanisms for their normal physiology. Therefore, researchers prefer to circumvent rather than directly inhibit MDR mechanisms. Developing anticancer drugs that are poor substrates for ABC transporters might be a good strategy in cancer therapy. Another approach is to prevent formation of new blood vessels which is called angiogenesis. For a long time, it is believed that tumors induce angiogenesis to provide an adequate blood supply for oxygen and nutrients. New vessel formation are inhibited using anti-angiogenic factors. However, most of the anti-angiogenic factors have other effects on the cells which limit their treatment. A major dose-limiting toxicity factor for anticancer drugs is to avoid complete eradication of bone marrow stem cells. Gottesman et al. produced multidrug resistant bone marrow cells by transfecting them with vectors carrying the MDR1 cDNA. This procedure allowed them to apply a chemotherapeutic regimen at otherwise unacceptable doses, and thus overcoming MDR [57, 117].

Recent studies suggest that a mutant ABCG2 protein is an ideal candidate for human stem cell protection and for use as a selectable marker in gene therapy. The cDNA encoding this protein is relatively small (about 2 kb) and the active dimer is spontaneously formed in the overexpressing cells. Since the R482G variant of ABCG2 has different substrate specificity than the wild-type protein, this mutant has a special advantage in gene therapy applications [126].

The exact mechanism of MRP1 involved multidrug resistance has not been clarified yet, though glutathione (GSH) is likely to have a role for the resistance to occur. N-acetylcysteine (NAC) is a pro-glutathione drug. DL-Buthionine (S,R)-sulfoximine (BSO) is an inhibitor of GSH synthesis. Recently, it has been investigated the effect of NAC and BSO on MRP1-mediated vincristine and doxorubicine resistance in human embryonic kidney (HEK293) and its MRP1 transfected 293MRP cells. Human embryonic kidney (HEK293) cells were transfected with a plasmid encoding whole *MRP1* gene. Both cells were incubated with vincristine and doxorubicine in the presence or absence of NAC and/or BSO. N-acetylcysteine increased the resistance of both cells against vincristine and doxorubicine. In contrast, BSO decreased NAC-enhanced MRP1-mediated resistance, indicating that induction of MRP1-mediated resistance depends on GSH. The results indicate that NAC and BSO have opposite effects in MRP1 mediated vincristine and doxorubicine resistance and BSO seems a promising chemotherapy improving agent in MRP1 overexpressing tumor cells. underlying the potential anticancer action of plant flavonoids await further

elucidation. Further in vivo studies of these bioactive constituents is necessary in order to develop flavonoid-based anticancer strategies. These studies demonstrate that antioxidants may have diverse effects in the cytotoxicity of chemotherapeutic drugs depending on their other pharmacological properties which may predominate their antioxidant effects [116].

TRANSCRIPTIONAL REGULATION OF ABC DRUG TRANSPORTERS

Herculean efforts over the past decade in the area of transcriptional regulation have shown that the fate of a gene is determined by the complexity and accessibility of a myriad of response elements within the promoter, as well as by the complement of transcription factors available to interact with these elements [76]. The composition of these factors is influenced by both the intra- and extracellular milieu, which can vary tremendously during the life of the cell. Thus, dynamic multiprotein complexes form, the nature of which is grossly dictated by promoter architecture yet subtly influenced by different signals, leading to profound regulatory switches. Superimposed upon this regulation is a dynamic chromatin ultrastructure, controlled by cofactors that transduce signals from promoter-bound proteins to the basal transcriptional machinery. Thus, it appears that each subset of genes is regulated by specialized multiprotein complexes that include both common basic components (the basal or general transcription factors), as well as unique components that tailor a complex for the transduction of signals initiated by particular developmental, metabolic or environmental stimuli.

CONSTITUTIVE (UNINDUCED) TRANSCRIPTION OF THE ABC TRANSPORTERS

Almost half of all genes transcribed by RNA polymerase II (Pol II) contain a TATA box. In promoters that contain this element, a first event is the recognition and binding of the TATA box by a general transcription factor, TBP. Through a series of protein–protein interactions, Pol II is recruited to the TATA element and initiates transcription 25–30 nucleotides downstream. How transcription is nucleated in promoters that lack the TATA box (TATA less promoters) is less clear, although many contain an initiator (Inr) element that encompasses the transcription initiation site and conforms to the consensus sequence PY-PY-A(+1)-N-T/A-Py-Py [127]. Although both the TATA box and the initiator element seem to serve similar functions with respect to recruitment of the transcription complex, some selectivity in protein requirements suggests a fundamental difference in the way these two classes of promoters are regulated. Interestingly, all of the human drug-related transporters examined to date lack an appropriately positioned TATA box (the human *MRP2* promoter was reported to contain a TATA-like element, but its location over 400 bp upstream of the transcription start site makes it functionally irrelevant), while several of their rodent homologues are TATA-dependent [76]. Transcription of *MDR1* is regulated instead by an Inr element, first identified through in vitro studies indicating that deletion of sequences downstream of +5 decreased elongation of correctly initiated transcripts to undetectable levels [128]. Transient transfection studies then defined the sequences between -6 and +11 as sufficient for proper initiation of

transcription *in vivo* [129, 130]. Although Inr's have not yet been functionally described in promoters of other drug transporters, examination of the published sequences identify consensus or near consensus Inr's within the promoters of the *MRP2* (GTACTTT) and *BCRP* (CCACTGC) genes [76].

GC BOXES AND CCAAT ELEMENTS

'GC' elements and 'CCAAT' boxes are among the most ubiquitous Pol II promoter elements and are found in the majority of TATA less promoters. Each element can interact with different families of proteins through sequence-specific DNA recognition. Since mutation or removal of these elements often leads to a complete loss of transcription, the proteins that interact with these elements were initially referred to as constitutive or 'basal' transcription factors. However, this label can be misleading, since more recent studies have shown that these factors are often essential for mediating activation by exogenous agents, particularly those that regulate chromatin structure. Like most 'TATA less' genes, the *MDR1* promoter includes both an inverted CCAAT box (-79 to -75), that interacts with the trimeric transcription factor NF-Y [131] and a GC rich element (-56 to -43) that interacts with members of the Sp family of transcription factors, specifically Sp1 and Sp3 [132, 133]. Transfection analysis of promoter constructs mutated in one or both of these elements indicate the requirement for each element in the constitutive (i.e. operative under normal growth conditions) expression of *MDR1* in some cell lines. Interestingly, early studies suggested that the YB-1 protein, a gene regulatory protein that preferentially interacts with RNA and single stranded DNA, specifically interacts with the *MDR1* inverted CCAAT box to mediate transcription [76]. However, YB-1 does not interact with double stranded oligonucleotides containing the *MDR1* CCAAT box; indeed, it interacts only with a single stranded oligonucleotide containing this element. Moreover, mutations within the *MDR1* CCAAT box that abolish transcription and NF-Y binding have no effect on the interaction of YB-1 with the single-stranded oligonucleotide [131], strongly suggesting that NF-Y, not YB-1, is the factor regulating *MDR1* through the CCAAT element. Nevertheless, although a direct involvement of YB-1 in MDR1 transcription appears unlikely, a number of studies have linked the expression or nuclear localization of YB-1 with an increase in *MDR1* [134, 135] as well as *MRP1* expression [136]. Whether YB-1 is activated in parallel with drug transporters as part of a global stress response or whether there is a direct non transcriptional role of YB-1 in their regulation remains to be determined. Upstream within the *MDR1* promoter (-110 to -103) lies an other GC element that is incapable of interacting with Sp1 [133], but may interact with another member of the Sp family or the highly related Kruppel factor family of transcription factors [137]. Immediately downstream and overlapping this GC-rich region is an inverted MED-1 element (-105 to -100) (multiple start site element downstream 1; iMED) that was first described in the hamster *MDR1* orthologue, pgp1, and shown to be involved in the activation of that gene in drug-resistant cells [138-140]. Functional disruption of iMED, either through mutation of the element or the use of a transcriptional decoy to sequester iMED-binding proteins, results in a decrease in *MDR1* transcription, although a role for this element in resistance-specific activation of the human homologue has yet to be established. GC elements are also important for constitutive expression of a number of other drug transporters. GC elements within the *MRP1* promoter (-91 to +103) are essential for optimal activity, and Sp1 has been shown to interact with these elements [141]. The *MRP3* proximal promoter contains several GC elements (-86 to -21) that

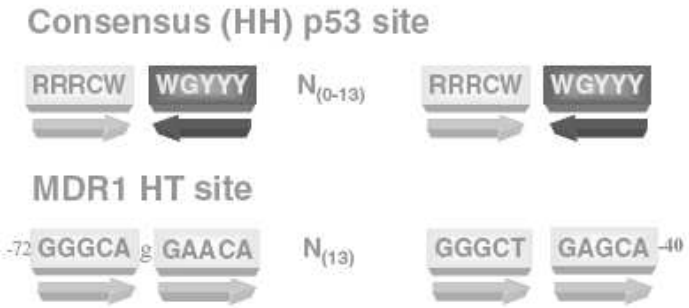
have been shown to interact with Sp1; removal of this region decreases *MRP3* transcription [142]. The *BCRP* promoter is TATA less and contains several putative Sp1 binding sites; sequences 300 bp upstream of the transcription start site are sufficient to confer basal promoter activity ([143]. Two functional GC boxes have been identified in the *ABCA2* gene; these elements interact with Sp1, Sp3 and the brain-specific Sp4 family member [144]. Interestingly, the proximal promoter of *MRP2*, although TATA less, appears to lack a proximal promoter GC element; a consensus Sp1 site has been identified at position –1709, but has not yet been functionally tested. *MRP2* does contain a putative CCAAT box that apparently interacts with YB-1 rather than NF-Y in vitro but this element is not required for constitutive transcription [145].

TUMOUR SUPPRESSORS,ONCOGENES AND *MDR1* TRANSCRIPTION

High levels of expression of multidrug transporters are often observed in drug-naıve tumours, even when the tissue of origin exhibits little or no expression of the corresponding gene. Hence, constitutive MDR1 or MRP gene expression is likely regulated in some cells by components that are involved in malignant transformation. It has been well established that tumours develop as a result of both uncontrolled proliferation and an intrinsic ability to escape cell death, mediated by the altered expression of various oncogenes and tumour suppressor proteins. Recently, it has become apparent that altered expression of several growth and death controlling proteins can adversely affect drug therapy in two ways: (1) by altering the cell's ability to respond to death signals and (2) by influencing the transcription and thus the expression of drug-resistant genes.

p53

The first evidence that a tumor suppressor protein could influence the expression of a drug-resistant gene came from the observation that wild-type p53 repressed transcription of the *MDR1* gene [146, 147]. Although a number of mechanisms had been proposed for this repression, this repression is mediated by a direct interaction of p53 with a novel binding element within the proximal *MDR1* promoter (-72 to -40) (Fig. 8) [148], making *MDR1* the prototype for a new class of p53-repressed promoters. Binding of p53 to this element, termed the HT (head-to-tail) site, appears to induce a novel tetrameric conformation of p53 that converts p53 from an activator to a repressor, perhaps through the differential recruitment of cofactors [76]. Repression by wild-type p53 has also been reported for the mouse and hamster *MDR1* homologues, while other studies suggest an activating role of p53 on the murine *mdr1* promoter in response to DNA damage and stress [76]. Wild-type p53 has also been shown to repress transcription of the human *MRP1* promoter [149, 150] and loss of p53 expression is correlated with increased *MRP1* expression in colorectal cancer [76]. Although the mechanism mediating the repression of *MRP1* has not yet been defined, there is some indication that it may involve deactivation of promoter-bound Sp1 [149].



Fugure 8. Schematic of the consensus head-to-head (HH) p53-binding site shown to mediate activation of target genes by p53 (top) and the non consensus head-to-tail (HT) site found within the MDR1 promoter and shown to bind p53 and mediate the repression of MDR1 transcription.

Paradoxically, several common mutant p53 proteins are able to activate, rather than repress, the *MDR1* promoter [146]; at least one of these mutants activates *MDR1* through a cooperative, and apparently mutant-specific, interaction with the Ets-1 transcription factor at a binding site within the proximal promoter region (-69 to -63) [151]. This type of direct effect has not been observed with the *MRP1* promoter, although it is reasonable to consider that mutations of p53 that inhibit its ability to repress *MRP1* transcription could be viewed as indirect 'activators'. The role of p53 in the regulation of drug resistance genes is not without controversy. Indeed, there are a few cases where opposing effects were observed in different cells or under different conditions. Therefore, the complexity of these systems should not be underestimated, and it is important to keep in mind that the intricate architecture of the individual promoters, the complement of endogenous p53 (mutant or wild type), the presence or absence of other p53 family members, as well as variations in cell- and tissue-specific co-effectors of p53 activity are all likely to influence the ultimate transcriptional readout in a given cell, tissue or tumor type.

Fos/Jun (AP-1)

There is some evidence, albeit indirect, that the AP-1 complex may be involved in the transcription of several drug transporters. AP-1 is the general term for transcription factor complexes composed of members of the Fos and Jun oncogene families [152]. AP-1 is constitutively expressed in many cell types, and DNA binding by the complex is induced by serum stimulation, phorbol esters and a variety of growth factors; it is also induced by various stress stimuli. Elevated levels of c-Fos have been demonstrated in a number of drug-resistant cell lines when compared to their drug-sensitive counterparts. Inhibition of PKA, an inducer of the AP-1 complex, was found to decrease the expression of human *MDR1* in the P388 leukemia cell line [153]; however, PKA has also been implicated in regulation by Sp1, complicating the interpretation of these data. A similar line of circumstantial evidence comes from studies of the c-Jun NH2-terminal protein kinase (JNK), which also activates AP-1. In human KB-3 cells, adriamycin, vinblastine and etoposide (VP-16) activate jun kinase (JNK), and this was found to be associated with an increase in *MDR1* expression at the mRNA level [76]. Two multidrug-resistant variants of KB-3, KB-A1 and KBV1, showed increased basal levels of JNK activity when compared to the KB-3 parental cell line. Putative non consensus AP-1 binding sites have been reported in human and rodent class I *P-GP* promoters. While the AP-1 site in the murine

homologue mediates the repression of this gene, the AP-1-binding elements in the promoters of the hamster (-55 to -49) and human genes (-121 to -115) are involved in transcriptional activation [76]. Moreover, cells transfected with c-Jun exhibit a higher level of expression of *MDR1* RNA and P-gp protein. The *MRP1* promoter also contains a putative AP-1 site (-498 to -492) that interacts with a complex containing c-Jun and JunD [154]. Interestingly, levels of this complex were increased in highly resistant H69AR cells as compared to their parental counterparts, although the role of this element in the regulation of *MRP1* in response to inducers has yet to be evaluated fully. Similarly, the *MRP2* promoter contains a consensus AP-1-binding element that has not yet been functionally tested [155].

Ras/WT-1

The MDR1 gene is also a target of the ras/raf signalling pathway [156]. Initial studies indicated that signaling by ras converges on the GC-rich binding site for the zinc finger transcription factors Sp1 and Sp3, located within the proximal *MDR1* promoter [156]. Egr-1, a ubiquitous immediate early factor, and WT-1, the Wilms' tumor suppressor protein, also interact with GC sequences through their zinc-finger domains. Interestingly, both Egr1 and WT-1 recognize a site within the *MDR1* GC element that overlaps the Sp1/Sp3-binding sequence. Indeed, activation of *MDR1* by TPA is mediated by Egr-1 [76] and suppressed by WT-1 [76]. A similar GC element resides within the promoters of several *MRP* genes, suggesting a potential role for the Ras pathway and WT-1 in their regulation as well. It appears, therefore, that the regulation of the expression of *MDR1*, and perhaps other drug transporters, by Ras/Raf and WT1 involves a complex interplay of transcription factors within a very discrete promoter region. To add to this complexity, it has recently been shown that the activation of phospholipase C (PLC) by a variety of inducers activates *MDR1* transcription through an upstream element (-106 to –99). This activation is enhanced by co-expression of constitutively active v-raf and blocked by a dominant-negative form of raf or by inhibitors of the mitogen-activated protein kinase, suggesting that PLC delivers the Ras/Raf signal to the *MDR1* promoter through a distinct binding site [76].

APC

Mutations in the tumour suppressor gene adenomatous polyposis coli (APC) have been documented in greater than 80% of all sporadic hereditary colon cancers. Loss of APC function leads to the nuclear accumulation of β-catenin, a co-activator for the transcription complex TCF/LEF (T-cell factor/lymphoid enhancer factor). A recent study showing that TCF/LEF activates the *MDR1* promoter provides one possible explanation for the overexpression of *MDR1*, and subsequent 'intrinsic' resistance, in many colorectal cancers [157]. In initial studies, the authors identified several TCF/LEF sites lying between –1813 and –261 within the *MDR1* promoter. Recently, they have extended their observations to the murine *MDR1* homologues by analysing mdr1a expression in Min/+ mice, which contain a mutant allele of the murine APC locus encoding a nonsense mutation at codon 850. Like humans with germline mutations in APC, Min/+ mice are predisposed to intestinal adenoma formation. Using this model, they have observed aberrant induction of the Mdr1a gene product, even in nascent microscopic adenomas of Min/+ mice. Interestingly, Min/+ mice devoid of the mouse

mdr1 homologues developed significantly fewer intestinal polyps, suggesting a role for P-gp in their genesis [158].

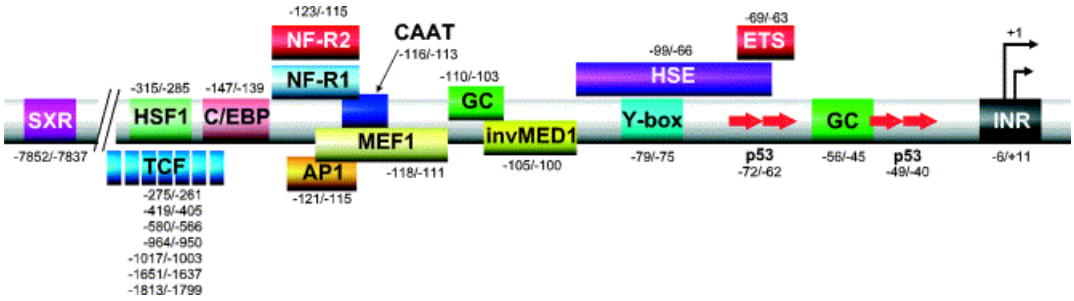


Figure 9. Promoter elements and DNA-binding factors involved in the transcriptional regulation of *MDR1*.

CONSTITUTIVE OVEREXPRESSION IN DRUG-RESISTANT CELLS

Tumor cell lines selected for resistance to MDR drugs most often exhibit constitutive overexpression of MDR1 or one of the other MDR-associated transporters. Although frequently the result of gene amplification, this overexpression can also be mediated at the level of transcription, particularly in the case of the MDR1 gene. Any drug-selected mutations/ alterations in any of the factors/pathways that are involved in regulation of the transporters could result in constitutive up-regulation, and future studies are likely to identify other mechanisms in other cell types.

MED-1/IMed

A mechanism described for the constitutive increase in the transcription of a *P-GP* gene was defined for the hamster p-gp1 homologue. Drug sensitive cells utilize a single transcription start site, while drug resistant cells initiate transcription from several downstream sites [138, 140]. Activation of the additional start sites is mediated by a novel downstream element, GCTCCC/G, designated as MED-1 (multiple start site element downstream) [139]. The MED-1 element interacts with a multiprotein complex that is currently under investigation and functions within a chromatin complex. Interestingly, it has also identified an inverted MED-1 complex, that we had termed iMED, upstream of the initiation site of the human *MDR1* promoter [76]. Mutation of iMED decreases transcription in drug sensitive cells, suggesting a function of this element in constitutive MDR1 transcription. Although its role in drug-resistant cells has not been directly investigated, a recent study suggests that binding of one of the MED-1 (iMED) complex partners may be increased in drug resistant leukemia cells [159], possibly linking iMED to the activation of *MDR1* in certain drug-selected cells. Interestingly, several other drug transporters, including MRP1 and MRP2, also utilize multiple start sites; whether a MED-1-like element is involved in their regulation is under investigation. MEF-1, a 130-kDa protein, termed *MDR1* promoter-enhancing factor 1 (MEF1), has been shown to activate *MDR1* transcription through an upstream promoter element (-118 to -111). Significantly, this protein was present in a drug-

resistant HL60 subline, but not in the drug sensitive HL60 parent cells [160], thereby suggesting a direct role in *MDR1* activation following drug selection. An interesting correlate has been found in MCF-7 cells, where it appears that the same promoter element binds an inhibitory complex containing NF-kB and c-Fos in parental cells, but not in resistant MCF-7/ADR cells [160]. The mechanism by which MEF1 or iMED regulates *MDR1* transcription, the interplay between MEF1 and NF-kB/c-Fos and the frequency with which these complexes are involved in *MDR1* activation in drug resistant cells remain to be determined.

STRESS INDUCTION OF DRUG RESISTANT GENES

Given the role of MDR1 in protection against environmental adversity, it is not surprising that the *MDR1* gene is highly responsive to stress signals [76]. *MDR1* inducers include heat shock, partial hepatectomy, inflammation, exposure to carcinogens including chemotherapeutics, hypoxia and UV and X irradiation. The effect of these inducers on the transcription of other drug transporters has just begun to be investigated. Heat shock activation of *MDR1* gene expression by heat shock and heavy metals was first reported by Gottesman and co-workers [161] and proposed to be mediated by a single mechanism. A heat-shock element (HSE) identified at position –152 to –178 was shown to interact with heat-shock factor (HSF); a second HSE like element (–99 to –66) bound to a protein complex which was largely unchanged upon heat shock [162]. However, the functional role of these elements in heat-shock response was not evaluated. More recently, it has been shown that activation by heat-shock requires the interaction of HSF1 with the –152/–178 HSE, while activation by other stress agents, including arsenite, butyrate and etoposide occur independent of thisHSE and HSF1 [163]. Indeed, activation by these and other stress inducers has been shown to occur through another promoter region, which we have termed the '*MDR1* enhancer'

INFLAMMATION (GR AND C/EBPb)

The 'acute-phase response' is a general term for the complex changes that take place in mammals in response to inflammatory stimuli such as bacterial infection or burn injury. This response is often experimentally simulated in rodents by the administration of bacterial lipopolysaccharide (LPS). In response to LPS, macrophages secrete inflammatory cytokines such as interleukin (IL)-1, IL-6 and TNF, which in turn act on the liver to induce a change in that organ's gene expression program, resulting in the synthesis of a range of acute-phase proteins [76]. Most of the analyses of the expression of drug transporters during inflammation have relied on the rodent model system; thus, data have been obtained primarily for the transcription of the rodent homologues. Although not necessarily directly applicable to the human genes, the high degree of promoter conservation among family members, together with the similar response of the human and rodent genes to inflammatory agents, suggests that similar transcription pathways exist. Under acute-phase conditions, *P-GP* genes are induced in the liver [164]. Interestingly, at least two transcription factors that are known to be induced during the acute-phase response have been shown to regulate *P-GP* gene expression in some cell lines. Studies carried out in a number of laboratories have shown that the IL-6-induced CAAT enhancer-binding protein (C/EBPb) can activate the mouse and human *MDR1* genes in transfection assays (-

147 to -139 in the human gene) [165, 166]. The homologous region in the hamster *p-gp1* promoter also contains a C/EBPb binding site and that activation through this site can be modulated by the binding of the glucocorticoid receptor (GR) [76]. These results suggest that this element may be important as a site of crosstalk between the inflammatory signals, mediated by cytokines through C/EBPb, and the anti-inflammatory signals, mediated by glucocorticoids through their receptor. The C/EBPb inducer IL-6 was also shown to induce *MRP2* expression and activity in human hepatoma cell lines [167]; the presence of a C/EBPb-binding site within the upstream region of the *MRP2* promoter (-356 to -343) suggests that this increase is mediated at the level of transcription [155]. A number of studies have suggested a role for glucocorticoids in the transcription of *MDR1* homologues, but the responses appear to be cell type specific. Using the mouse hepatoma cell lines Hepa 1–6 and hepa1c1c, it was found that the synthetic glucocorticoid, dexamethasone, elicited an increase in the expression of the two murine *MDR1* homologues, mdr1a and mdr1b [168]. Nuclear run-on analysis indicated that this increase occurred at least in part at the transcriptional level and could be abrogated by the protein synthesis inhibitor cycloheximide, suggesting that GR was influencing this promoter through an indirect mechanism. A similar increase in human *MDR1* RNA levels was observed in the HepG2 human hepatoma cell line. In rat primary hepatocytes, however, dexamethasone treatment led to a decrease in mdr1b expression, and no increase was seen in the non-hepatoma mouse LMtk- and NIH3T3 cell lines or in the human HeLa cell line upon dexamethasone treatment, suggesting that the effect is cell line specific. A glucocorticoid response element (GRE) has been identified in the promoter of the hamster *MDR1* homologue, *p-gp1*. This site, between -96 and -83, mediates the repression of pgp1 transcription by GR in both DC-3F Chinese hamster lung cells and a human osteosarcoma cell line, U2-OS. The GRE overlaps a binding site for CEBPb and it appears that GR represses *pgp1* transcription by interfering with the actions of CEBPb, as both sites are required for repression to occur. Interestingly, these elements are conserved in the human *MDR1* gene and *MDR1* transcription can also be repressed by GR in some cell types, suggesting that a similar mechanism may be involved. Treatment of rats with dexamethasone increases *MRP2* mRNA in vivo, although the mechanism underlying this increase has not yet been determined [169]. The fact that glucocorticoids are currently used in chemotherapy for many tumors, including chronic lymphocytic leukemias, lymphomas, multiple myelomas and breast cancers makes an understanding of the role of these hormones in the regulation of drug transporters an important goal.

HYPOXIA

The microenvironment of many large, rapidly growing tumours lacks a sufficient vascular supply, resulting in oxygen deprivation or hypoxia. Prolonged hypoxia has been linked to metastasis, since it increases genomic instability, genomic heterogeneity, and may act as a selective pressure for tumour cell variants [76]. This hypoxic environment results in the induction of many stress–response genes, including glycolytic enzymes, pro-angiogenic factors and pro-inflammatory genes. Apropos of this, it has recently been shown that *P-GP* expression is increased in hypoxic cells, and that this increase is mediated by hypoxia-inducible factor-1 (HIF-1), a transcription factor that normally resides in the cytoplasm of normoxic cells and is believed to be shuttled to the nucleus upon hypoxic stress. HIF-1 activates the *MDR1* promoter through a consensus binding sequence (5'GCGTG3'; -49 to -45) that

overlaps the GC element involved in constitutive and inducible expression. Indeed, preliminary evidence suggests that the GC-binding protein Sp1 may be involved in the hypoxic response. These results may in part explain the observation that hypoxic tumour cells are more refractory to anticancer agents [76].

CARCINOGENS

Most early studies on the effects of carcinogens on *P-GP* expression concentrated on the rat genes. These genes are induced by a number of xenobiotics including 2,3,7,8-tetrachlorodibenzo-p-dioxin, 2-acetylaminofluorene (2-AAF) and 3-methylcholanthrene [76]. The transcriptional mechanism underlying this activation was elusive until it was shown that activation of rat mdr1b by 2-AAF involves an NF-kB-binding site at position -167 to -158 within the promoter. 2-AAF was found to activate *mdr1b* through the generation of reactive oxygen species, leading to the activation of IkB kinase, the degradation of IkB beta and the activation of NF-Kb [170]. A different mechanism has recently been shown to mediate hepatic induction of the human *MDR1* gene by 2-AAF. In this case, the carcinogen activates phosphoinositide 3-kinase and its downstream effectors Rac1, NAD(P)H oxidase and Akt, resulting in activation of *MDR1* through an upstream binding site (-6092) that includes an NF-kB-binding site [171].

CHEMOTERAPEUTICS

Considerable evidence has accumulated to indicate that the expression of drug transporter genes can be transiently induced in response to chemotherapeutics [172-174]. This was first reported in CCRF-CEM/ ActD cells that exhibited an increased steady-state level of MDR1 RNA following short-term exposure to actinomycin D [173]; this increase was mediated, at least in part, at the level of transcription. Early studies indicated that the MDR1 promoter region from -136 to -76 was involved in activation by actinomycin D [175]; this region was further delineated to the *MDR1* enhancesome. Although it was initially assumed that only those drugs associated with the MDR phenotype would induce the expression of *P-GP* genes, more recent studies indicate that *MDR1* transcription can also be induced by non-MDR drugs, such as antifolates and hydoxyurea [76]. In this study, induction of *MDR1* was associated with morphological indications of cell damage, suggesting that increased *P-GP* transcription may be part of a general cellular response to damaging agents. Interestingly, some drugs, such as mitomycin C, suppress the activity of MDR1, although the mechanism by which this occurs has not been determined [176, 177]. It should also be noted that overexpression of *MDR1* in response to chemotherapeutics was shown to be a result of changes in mRNA stabilization and translational initiation in several leukemia cell lines, with no apparent transcriptional component [178]. Thus, it is likely that multiple mechanisms exist in different cell types that either cooperatively or exclusively regulate *MDR1* gene output. While few studies have focused on the regulation of other drug transporters by chemotherapeutic agents, *MRP2, MRP3* and *MRP5* were shown to be induced in liver HepG2 cells by cisplatin, although the mechanism underlying this induction was not investigated [179].

'MDR1' ENHANCEOSOME

It has been well established that *MDR1* gene expression can be activated by UV radiation, differentiation agents such as sodium butyrate and retinoic acid, HDAC inhibitors, phorbol esters and certain chemotherapeutics (Fig. 10). Recent studies in our laboratory indicate that the signals from all these divergent stimuli converge on a region of the *MDR1* promoter that we refer to as the '*MDR1* enhancesome' [131, 180]. This region includes binding sites for the trimeric transcription factor NF-Y and the Sp family of GC-binding transcription factors. Together, these DNA-binding proteins cooperate to recruit the histone acetyltransferase P/CAF to the *MDR1* promoter, resulting in the acetylation of promoter-proximal histones and subsequent transcriptional activation that is likely mediated by further chromatin remodeling. Recently, chromatin immunoprecipitation studies have identified a 'switch' in DNA-binding Sp family members following induction. Studies are presently underway to determine whether this change in binding factors results in recruitment of new co-activators/co-repressors to the *MDR1* promoter, and whether other factors that have been shown to bind to the *MDR1* GC element are also involved in stress response through the enhancesome complex. Although the mechanism by which each agent transduces the signal that results in promoter activation has yet to be determined, the role of the *MDR1* enhancesome in the regulation of transcription by a variety of stimuli makes it an attractive target for therapeutic intervention [181].

NUCLEAR RECEPTORS AND TRANSCRIPTION OF DRUG TRANSPORTERS

While many ABC transporters are ubiquitously expressed, high levels of expression are usually restricted to a limited number of tissues or cell types where they transcriptional regulation of ABC drug transporters perform a specialized function. One organ that depends on the action of ABC transporters to carry out its prescribed function is the liver. In addition to MDR1, which is involved in lipid transport and drug biodistribution and is activated during inflammation and partial hepatectomy of the liver, several other drug transporters are involved in liver function, including MRP2, MRP3 and BSEP (ABCB11). The major physiological role of MRP2 is to transport conjugated metabolite sinto the bile canaliculus, while MRP3 is localized in the basolateral membrane of the hepatocytesand transports similar metabolites back to the bloodstream. This may explain why mutation of MRP2 leads to Dubin–Johnson syndrome, characterized by elevated levels of bilirubin, increased urinary coporphyrin I fraction and deposition of dark pigment in the liver. Monoanionic bile salts are secreted into bile canaliculi by the bile salt export pump BSEP. Given the functional relationship among these transporters, it is not surprising that their transcription is regulated through similar mechanisms involving nuclear hormone receptors. Nuclear receptors are comprised of a family of transcription factors that function as heterodimers to regulate target promoters. RXR (retinoid receptor) is present in all heterodimers; the second partner determines the substrate ligand and the target promoters that will be activated. The nuclear receptor proteins that have been shown to be involved in transcription of drug transporters include retinoic acid receptor (RAR), farnesoid receptor (FXR) steroid-activated receptor (SXR; P(pregnane)XR in rodents) and constitutive androstane receptor (CAR). The RXR-containing heterodimers regulate a broad range of hepatic metabolic functions, including bile acid synthesis, fatty acid and oxysterol metabolism, and cytochrome

oxidase drug metabolism. RARα in complex with RXRα has been shown to regulate transcription of the rat *MRP2* promoter through a binding element located in the upstream promoter region (-422 to -398) (Denson et al., 2000); the cytokine IL-1β suppresses activation through this site. Although the *MDR1* promoter is activated in neuroblastoma cell lines by all-trans retinoic acid (ATRA), activation appears to be independent of RAR/RXR binding and is instead mediated by the differential binding of Sp family members to the GC element within the *MDR1* enhancesome [181]. FXR, when combined with RXR and bile salts as its ligand, initiates feedback control of bile acid synthesis [181]. Both *BSEP* and *MRP2* are targets of the FXR:RXR heterodimer. An FXR response element identified in the human *BSEP* promoter (-192 to -180) mediates the activation of *BSEP* transcription by bile salts; a similar element exists in the rat *BSEP* promoter (-64 to -52). Moreover, FXR-/- mice exhibit a dramatic decrease in the expression of *BSEP*, consistent with the role of this receptor in regulation of *BSEP* transcription. Notably, lithocolic acid, a potent inducer of choleostasis, decreases the transcription of *BSEP* by antagonizing the activity of FXR, implicating FXR and loss of BSEP in intrahepatic choleostasis [182]. An atypical promoter element has been identified within the rat *MRP2* promoter (-401 to -376) that interacts with FXR and mediates transcriptional activation. This binding site also mediates the transcriptional regulation of *MRP2* by PXR (SXR) (a receptor for xenobiotics such as rifampin, phenobarbital, taxol, clotrimazole and hyperforin) and by CAR [183]. Interestingly, the human *MDR1* promoter has also been shown to be regulated by the interaction of SXR:RXR with a promoter element ~8 kb upstream of the transcription start site [184, 185]. Taken together, these results suggest that activation of drug transporters through multiple nuclear receptors can alter the efflux, and therefore the pharmacokinetics and bioavailability of a variety of compounds, including chemotherapeutic agents. Notably, despite the presence of putative nuclear receptor binding sites within the *MRP3* promoter, regulation of *MRP3* by bile salts in enterocytes is mediated by the interaction of the alpha fetoprotein transcription factor (FTF) with the *MRP3* promoter (-229 to -138) [186]; how this promoter is regulated in liver cells is presently under investigation. In *MDR1* promoter several of the binding sites for transcription factors overlap, and in most cases it is not yet known whether multiple factors can co-occupy their cognate promoter site at the same time, or whether their interaction with the promoter is mutually exclusive. Keep in mind that most of the studies identifying these factors were performed in tissue culture cells and the relevance of these findings to the in vivo situation, particularly in the clinical setting, remains to be determined.

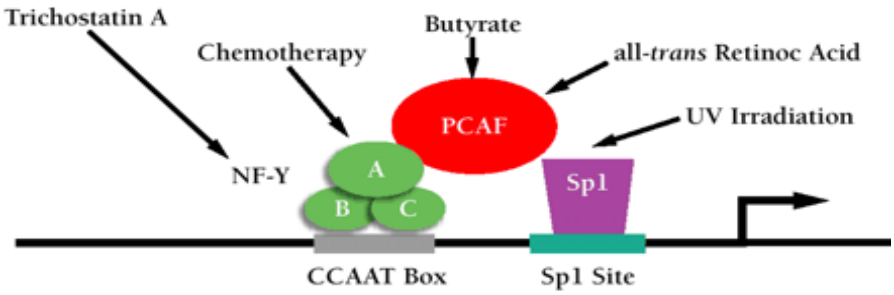


Figure 10. A variety of environmental signals, including those induced by hormones (ATRA), radiation, HDAC inhibitors (TSA, butyrate), some chemotherapeutics, phorbol esters and others converge on the *MDR1* enhanceosome, which includes the DNA-binding proteins Sp1, Sp3 and NF-Y, the histone acetylases P/CAF and P/300. depending on conditions, the transcription factors Egr1, WT-1 and the co-repressor HDAC1 may also be found at the promoter.

TARGETING TRANSCRIPTIONAL REGULATION OF ABC TRANSPORTERS GENES.

p53

The frequent occurrence of p53 mutations in human cancer has led to numerous investigations evaluating its role as a potential therapeutic target. By restoring wt p53, investigators have sought to either: (1) revert the malignant phenotype or (2) enhance drug sensitivity. These goals are based on available evidence that although not conclusive suggests that: (1) restoration of p53 will have an impact on the malignant phenotype and (2) mutant p53 confers drug resistance. Numerous investigators have shown that levels of p53 above a certain 'threshold' almost invariably lead to apoptosis or to enhanced drug sensitivity [187, 188]. Several studies have shown that wt p53 can be introduced using viral vectors, and the available reagents can achieve the 'threshold' levels required to bring about apoptosis. Studies to date have employed conventional vectors and also explored innovative strategies to exploit the p53 status of cancer cells. The first clinical trials attempting to restore wt p53 function utilized retroviral vectors that constitutively expressed p53 under the control of the actin promoter [189]. Following intratumoral injection, expression of p53 and enhanced apoptosis were observed in the injected tumors, and in three of nine patients limited evidence of tumor regression was noted. While the retroviral vector used in these studies proved to be inefficient, this study provided a first 'proof of principle', and became the catalyst for further studies. Subsequent studies have used adenoviral vectors. Advantages of the latter include the ability to infect cells in all phases of the cell cycle, relative ease of growth to high titers and the capacity to accommodate inserts of large size. Its major disadvantages include the frequent occurrence of antibodies in the general population, preferential but not exclusive infection of cells expressing the coxsackie adenovirus receptor (CAR) and au-integrin and its lack of selectivity, so that it infects normal cells, most notably liver, with high efficiency [190]. Despite its limitations, Adp53 vectors appear to be well tolerated and expressed in most patients. Clinical trials have shown antitumor activity in patients with squamous cell carcinomas of the head and neck and nonsmall- cell lung cancer (NSCLC) [190]. In evaluating these strategies, one must be mindful that p53 participates in regulating the expression of a wide range of genes with diverse functions and that some of these genes may have unanticipated roles in a vector's efficacy. Thus, some of the genes expressed following infection with a p53-expressing adenovirus may modulate a 'bystander effect' by which uninfected cells are damaged as a result of the expression of genes in infected cells. For example, p53 may indirectly regulate angiogenesis by modulating the expression of vascular endothelial growth factor (VEGF) and thrombospondin [190]. p53 also participates in the expression of insulin-like growth factor 1-binding protein (IGF1BP), an antagonist of insulin-like growth factor 1 (IGF-1), a growth factor implicated in tumor cell survival [190].

YB-1

The Y-box-binding protein YB-1 has multiple functions, including regulation of gene expression. Nuclear localization of YB-1 regulates *MDR1* and *MRP1* gene expression that is known to confer the multidrug resistant phenotype in tumor cells [191]. YB-1 is involved in several aspects of drug resistance, and it is an important regulator of pleiotropic resistance [192]. *YB-1* overexpression with increased nuclear localization occurs in many malignant diseases (e.g., breast, non–small cell lung, osteosarcoma, ovarian, and colon carcinoma; refs. 19–23). Furthermore, *YB-1* mRNA is highly abundant in glioblastomas and malignant melanomas and is involved in the progression of prostate cancer [193]. Several studies indicate that YB-1 positively regulates cell proliferation and it was shown that YB-1 translocates from the cytoplasm to the nucleus at the G1-S-phase transition [194]. Recent evidence suggests that activated Akt is involved in nuclear translocation of YB-1 and blocking of phosphatidylinositol 3-kinase/Akt pathway may therefore be helpful for overcoming chemoresistance [195]. The use of replication-competent adenoviruses for cancer therapy receives widespread attention, especially for the treatment of tumors insensible to current treatments. YB-1 facilitates E1-independent adenoviral replication by targeting the adenoviral E2-late promoter in multidrug-resistant cancer cells [196]. In addition, the adenoviral protein E1B55k is involved in nuclear translocation of YB-1. Given that complexes containing adenoviral proteins E4orf6 and E1B55k play critical roles in productive infection as well as in nuclear translocation of YB-1, targeting YB-1 by a recombinant E1/E3-deleted adenoviral vector expressing E1B55k and E4orf6 will be capable of translocating YB-1 into the nucleus and in consequence to replicate and destroy tumor cells. E2-early is only needed in little amounts at the very early state of infection, whereas E2-late acts throughout the whole cycle in quite considerable quantities. The E2-early promoter is active at early times postinfection [193]. Because YB-1 acts through the E2-late promoter, it represents a suitable target for oncolytic adenovirus development. E1A mutant adenoviruses with reduced S-phase induction show tumor-selective replication [193]. Adenoviral expression of E1B55k and E4orf6 mediates nuclear translocation of YB-1 in tumor cells. Nuclear translocation of YB-1 only takes place when cells are infected with WT adenovirus or Xvir03 but does not occur with an E1B55k-deleted adenoviral vector or an E1-deleted adenoviral vector (and therefore lacking E4orf6 expression) expressing E1B55k under CMV control [193]. Conditionally replicating adenoviruses have shown enhanced anti-tumour activity when combined with chemotherapy or radiation and it has been suggested in several reports that irradiation or chemotherapy creates an environment that is more conductive to adenoviral infection or replication, including our own data showing that cytostatic drugs cause an increase of nuclear YB-1 and concomitant viral replication [193]. As mentioned earlier, YB-1 regulates, besides other factors, the expression of the drug-related transport proteins MDR1 and MRP1. YB-1-associated oncolytic adenovirus Xvir03 causes inhibition of these genes. Xvir03 inhibits the expression of multidrug resistance–related genes *MDR1* and *MRP1*. Because both genes are regulated by YB-1, this indicates that the recruitment of YB-1 via the complex E1B55k/E4orf6 to the adenoviral E2-late promoter is responsible for this effect. This is supported by the observation that the expression level of *MDR1* was unaffected by an E1-deleted adenoviral vector expressing E1B55k under CMV control. Nuclear translocation of YB-1 by Xvir03 leads to resensitization of tumor cells to cytostatic drugs; thus, the potential of radiation and chemotherapy is restored. Chemotherapy,

on the other hand, yields an increased expression and nuclear localization of YB-1 and in consequence enhances YB-1-associated adenoviral replication.
This reveals a link between chemotherapy and virotherapy based on the cellular transcription factor YB-1. Xvir03 is to our knowledge the first oncolytic vector that possesses as a single agent the potential of down-regulating two multidrug resistance–related genes *MDR1* and *MRP1*. Due to their low homology, it is unlikely that a potent, nontoxic inhibitor for both proteins could be developed. It is possible propose a model termed Mutually Synergistic Therapy (MUST), which highlights the existing relationship between YB-1-associated virotherapy on the one hand and chemotherapy on the other hand. Thus, combining YB-1-dependent virotherapy with chemotherapy is beneficial for both treatments. This theoretical model may be helpful in developing new combined strategies involving YB-1-associated virotherapy for cancer intervention to augment the effectiveness of cytotoxic drugs and extend patient survival [193].

THE RISE OF DNA METHYLATION AND THE IMPORTANCE OF CHROMATIN ON MULTIDRUG RESISTANCE IN CANCER

Thus far, we have discussed transcription as the interaction between transcription factors and their cognate DNA binding sites. However, superimposed upon the regulation mediated by those interactions is the role of chromatin in permitting this interplay to occur. The basal transcriptional state of chromatin is inactive – the DNA wrapped in a nucleosomal complex is generally inaccessible to transcription factors. However, chromatin is a dynamic structure that receives signals from the environment to trigger changes in chromosomal architecture. A number of elegant studies have shown that covalent modifications of the amino-termini of the core histones in nucleosomes are critical to the regulation of transcription. These modifications, which include acetylation, methylation, phosphorylation and ubiquitination, appear to occur in sequential patterns, leading to the hypothesis that their intercommunication provides a ‘histone code’ that, when deciphered by other components of the transcriptional machinery, signals for transcriptional activation/deactivation [197]. Two classes of chromatin-targeted proteins have been identified that act as on/off switches for transcriptional competence: chromatin remodeling proteins and chromatin-modifying enzymes. Chromatin-modifying enzymes, specifically histone acetylases (HATs)/ histone deacetylases (HDACs) and DNA methylases, have been shown to be involved in the regulation of the *MDR1* gene (Fig. 11).
The silencing of gene expression is associated with deacetylated histones, while histone acetylation neutralizes the positive charge of the lysine-rich histone tails, thereby weakening the interaction of histones with the negatively charged DNA and generating an ‘open’ chromatin conformation. Moreover, acetylation of the transcription factors themselves can add an additional layer of regulation to this process. Histone acetylation is reversed by the action of the histone deacetylase (HDAC) family of chromatin-modifying enzymes. How these modifying enzymes are recruited to specific promoters at specific times is still under intensive investigation. However, a general model proposes that HDACs are recruited to the promoter by sequence- or modification-specific proteins, thereby maintaining the gene in a hypoacetylated, inactive state. In response to specific stimuli, transcriptional

activators recruit the HATs that acetylate histones, resulting in conformational changes within the nucleosomal array. Treatment of cells with the HDAC inhibitors trichostatin A or sodium butyrate leads to the activation of MDR1 transcription and associated hyperacetylation of MDR1 proximal-promoter histones [180]. This activation is mediated by the HAT protein, P/CAF, which is recruited to the promoter via its interaction with a transcriptional complex that we termed the *MDR1* enhancesome. Interestingly, activation appears to be limited to cells that already express detectable levels of *MDR1* RNA. In the few cell types in which the *MDR1* gene is silenced, altering the equilibrium of chromatin acetylation is not sufficient to activate transcription. Recent studies suggest that this may be the result of hypermethylation of the *MDR1* promoter, which acts as the ultimate 'lock-down' of gene expression.
The role of DNA methylation in transcriptional silencing is due to the capacity of methylated DNA to assemble repressive chromatin as compared with conventional unmethylated chromatin. An increasing body of evidence indicates that DNA methylation significantly contributes to the activation and repression of many different genes, including *MDR1* [76]. CpG methylation alone is not sufficient to instantly confer repression [198, 199]. Rather, the targeting and sequences of repressive complex specifies a transcriptional block. Indeed, chromatin accessibility studies demonstrated that DNase hypersensitivity was reduced in a cell line where the *MDR1* promoter was hypermethylated, compared to cell line where the promoters were hypomethylated, indicating *MDR1* promoter methylation imposes a restrictive chromatin environment. The methyl-CpG-binding protein-2 (MeCP2) protein, a strong transcriptional repressor, was identified to localize to hypermethylated *MDR1* chromatin and was associated with methylation dependent silencing. MeCP2 localization was correlated with hypermethylation and *MDR1* transcriptional silencing, as MeCP2 was absent from the hypomethylated promoter in those cell lines that expressed *MDR1*.

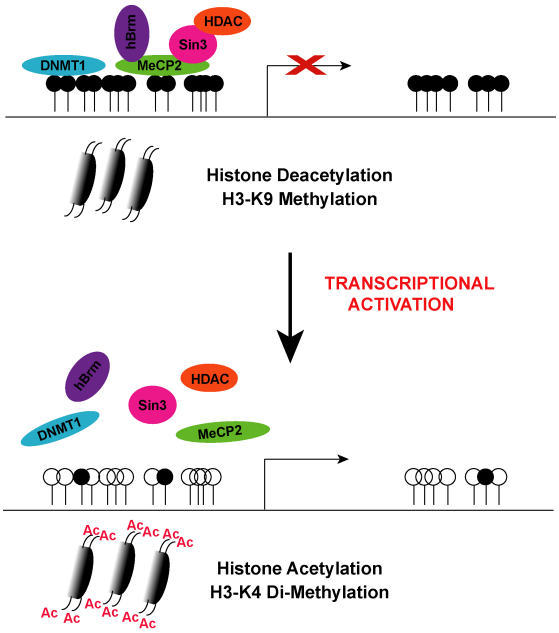


Figure 11. Hypothesized epigenetic models for *MDR1* activation.

Robust *MDR1* expression was only obtained when demethylation induced by 5-azacytidine treatment was accompanied with TSA treatment. MeCP2 silences

transcription by recruiting the co-repressor mSin3, Hdac1 and Hdac2 via an interaction with its transcriptional repressor domain (TRD). Treatment with TSA has been shown to partially overcome TRD-mediated repression. However, the failure of TSA to reactivate *MDR1* demonstrated that Hdac inhibition is not the only mechanism of repression associated with MeCP2. more recently, MeCP2 was shown to interact with a protein complex containing methyltransferase activity specific to histone H3 lysine residue 9 (H3K9). Loss of MeCP2 was shown to reduce the level of H3K9 methylation at the in vivo gene target , *H19*, implicating H3K9 methylation as a second mechanism, in addition to Hdac mediated repression, by which MeCP2 functions to repress gene expression. Interestingly, H3K9 methylation has also been shown to direct CpG methylation in Neurospora Crassa and Arabidopsis thaliana, implicating that the reverse association is also possible. However this reverse mechanism is yet to be identified in mammalian cells.

The histone modification, methylation of H3K9, is heavily associated with heterochromatin. Targeted disruption of the mouse homologues of the H3K9 methyltransferases, Suv39h1 and Suv39h2, resulted in disruption of pericentromeric heterochromatin and loss of H3K9 methylation. This was also demonstrated in yeast, as Clr4 (H3K9 methyltransferase) was shown to be necessary for transcriptional silencing at centromeric heterochromatin emphasizing the roles that H3K9 methyltransferases and H3K9 methylation play in establishing and maintaining heterochromatic states. Evidence also now indicates that H3K9 methylation is involved in regulating euchromatic gene silencing. H3K9 methylation was localized to regions of silenced developmentally regulated genes in chicken, and silenced tumour suppressor genes in cancer. Furthermore, dynamic changes in H3K9 methylation in response to gene activation have been demonstrated at several promoters. The human methyltransferase Suv39H1 and G9a, were targeted to the endogenous *VEGF-A* promoter, inducing gene silencing and thus demonstrating that H3K9 methylation can initiate silencing. H3K9 methylation is thought in part, to mediate repression by providing a docking site for the transcriptional repressor, HP1, and HP1 was later demonstrated to recognize and specifically bind methylated H3K9. loss of the H3K9 methyltransferase, Clr4 in yeasts, was shown to be necessary for transcriptional silencing at centromeric heterochromatin, and localization of Swi6, a HP1 homolgue. Whether HP1, or H3K9 methylation contributes to *MDR1* silencing is not clear. [200]

The *ABCG2* promoter in renal carcinoma was found to be methylated [201]. This can be of therapeutic importance because renal carcinoma cell lines UOK121 and UOK143, having a methylated *ABCG2* promoter and expressing a lower level of ABCG2, were found to be more sensitive to ABCG2 substrate drugs. Consistent with the role of DNA methylation in *ABCG2* silencing, incubation of methylated cell lines UOK121 and UOK143 with 5-aza-dC, a specific inhibitor of DNA methyltransferase, resulted in upregulation of *ABCG2* expression in a concentration-dependent manner. It has been suggested that DNA methyltransferases may act only on chromatin that is methylated at lysine 9 on histone H3 (H3K9) [202, 203]. Indeed, H3K9 methylation is sufficient for initiating a gene repression pathway in vivo [204]. Modifications (methylation and deacetylation) of histone H3 assembled at the *ABCG2* promoter and DNA methylation of the CpG island coordinately cause silencing of the *ABCG2* gene. The *ABCG2* expression in the methylated cell lines (UOK121 and UOK143) can be restored by 5-aza-dC treatment, and the reactivation is associated with hyperacetylation of H3 at lysine 9. Thus, apart from demethylating the *ABCG2* promoter, 5-aza-dC also resets the histone code, switching it from methylation to acetylation at H3K9. MBD proteins mediate silencing of genes by facilitating the

establishment of a repressive chromatin environment [205, 206]. These proteins recruit chromatin remodeling enzymes such as histone deacetylases and mSin3 to the DNA with their transcriptional repression domains, creating an inactive chromatin configuration [207-209]. ChIP assays demonstrated binding of MeCP2, MBD2, HDAC1, and mSin3A to the CpG island region in UOK121 and UOK143 [201]. ChIP analyses also revealed that 5-aza-dC treatment in UOK121 and UOK143 cells facilitated an enrichment of acetyl-H3, a release of MBDs (i.e., MeCP2 and MBD2), and a decreased occupancy of HDAC1 and mSin3A on the *ABCG2* promoter, consistent with a more open chromatin conformation that would allow transcriptional activation. Taken together, the data support the notion that DNA methylation dependent formation of a repressor complex in the CpG island contributes to inactivation of the *ABCG2* gene [201]. The binding of the MBDs to methylated DNA results in recruitment of HDACs to support transcriptional repression. The HDAC inhibitor depsipeptide could upregulate *ABCG2* expression most effectively when it is unmethylated but to a lesser extent when is heavily methylated. This suggests that DNA demethylation is more critical than histone acetylation for the *ABCG2* gene chromatin to switch from a transcriptionally non permissive to a permissive configuration. In this regard, the repressive histone code, MeH3K9, was found binding strongly to the proximal *ABCG2* promoter in the methylated cell lines, which was not affected by depsipeptide treatment [201]. In contrast, depsipeptide did reduce the association of MeH3K9 with the *ABCG2* promoter in the unmethylated cell line. This may explain why depsipeptide has less effect on upregulation of *ABCG2* in the methylated cell lines than in the unmethylated cell line [201]. A similar finding has been reported for *FMR1* transcriptional silencing in fragile X cells [210].

DNA methylation can confer a selective growth advantage to cells when it occurs in the promoter regions of genes repressing the expression of tumor suppressor genes, resulting in the development of cancer [211, 212]. Since ABCG2 normally functions as an efflux transporter, the physiological significance of this methylation-dependent repression in cancer is not clear.

Whether the repression of *ABCG2* would provide an advantage to the cell, or merely be an epiphenomenon, is open to speculation. Regardless of whether ABCG2 repression could be of benefit to cancer cells, the identification of patients whose tumours have repressed ABCG2 could be important. Drugs that are substrates for ABCG2 would be expected to be more effective in such a patient population. A more important role of *ABCG2* methylation could be that in normal physiology. Gene methylation is believed to be the basic mechanism for the establishment and maintenance of genomic imprinting [213]. Imprinted genes are marked in the male and female germ lines and retain the molecular memory of their parental origin, resulting in allelic expression differences. However, gene regulation at the promoter level during normal growth and development is not well understood. Furthermore, *ABCG2* is normally expressed in the placenta and in stem cells [88, 101]. The possible role of promoter methylation in regulating *ABCG2* expression in stem cells and their progeny has not been evaluated. Since it would require isolation of different populations of cells, including stem cells, progenitor cells, and terminally differentiated cells, this pursuit must await progress in the identification of specific markers for cells at different stages of development.

The role of chromatin in the regulation of other drug transporters has not fully been investigated. Activation of the *MRP7* promoter by TSA has also been observed, although the mechanistic basis of this activation has not been elucidated [76]. Interestingly, in HepG2 cells, TSA repressed transcription of the *MRP2* gene through an upstream region (-517 to -197), suggesting that chromatin modification may

permit binding of a repressor complex to this promoter [76]; TSA had no effect on the expression of *MRP3* in these studies.

CHEMOTHERAPY CAN INDUCE LOSS OF METHYLATION

A dominant role for CpG methylation in *MDR1* silencing indicates that the methylation mark may need to be removed in order to acquire Pgp mediated MDR. Long term selection for drug resistant cell lines was associated with hypomethylation of the *MDR1* promoter, however the extended kinetics of the exercise suggested that selection of hypomethylated drug resistant clones was the primary mechanism for the loss of methylation. Several studies have shown that demethylation of the *MDR1* promoter does occur during the course of clinical chemotherapy, resulting in the activation of *MDR1* expression. This raised the intriguing question of whether the cytotoxic action of chemotherapeutic drugs could induce active demethylation of the *MDR1* promoter. Alternatively, methylation may be a permanent mark of the *MDR1* promoter, and instead, the observed loss of methylation may have been due to the selection of cells with a reduced methylation pattern, and hence, resulted in increased *MDR1* expression following drug treatment. A protein with demethylase activity has not been conclusively identified that can reverse DNA methylation, even though evidence exists that demethylation does occur at some genes over time.
MBD2, a methyl-binding domain protein associated with methylation dependent silencing, was demonstrated to have demethylase capabilities that were promoter and time dependent. However similar activity for MBD2 were not demonstrated in other studies, therefore, there is only weak evidence to support a scenario whreby chemotherapeutic drugs coul direct a putative demethylase activity to the MDR1 promoter to reverse the methylation [200].

THE Myc/Max/Mad NETWORK AND THE TRANSCRIPTIONAL CONTROL OF CELL BEHAVIOR

The Max network comprises a group of nuclear transcription factors whose functions profoundly affect cell behavior [214].These factors possess two common attributes. First, members of the network are a subset of the larger class of proteins containing basic helix-loop-helix zipper (bHLHZ) motifs. This domain is known to mediate protein-protein interactions and DNA binding [214]. Second, each of the members of the network utilizes its bHLHZ domains to form individual dimers with Max, itself a small bHLHZ protein (Fig 12). Association with Max results in the formation of a heterodimer possessing sequence-specific DNA binding and transcriptional activities. Max itself can homodimerize and bind DNA, but such Max homodimers appear to be transcriptionally inert [215, 216]. The ability to modulate transcription is derived from specific domains within the Max interacting factors which, in turn, appear to mediate associations with specific

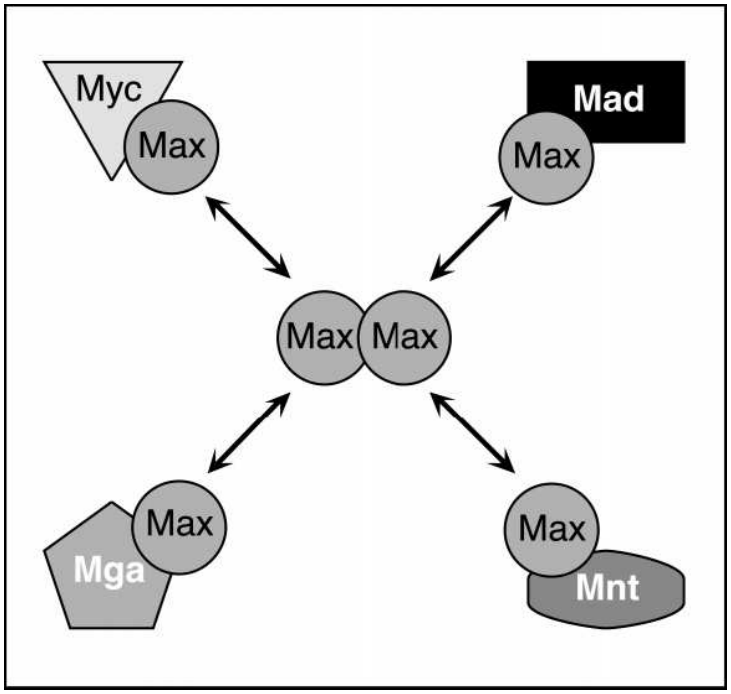


Figure 12. Max-interacting proteins. Max forms heterodimers with members of the Myc and Mad protein families as well as with the Mnt (or Rox) and Mga proteins. Each of these proteins interacts with Max through its bHLHZ domain.

coactivators or corepressors, resulting in the formation of higher-order complexes. Because Max-interacting proteins homodimerize poorly on their own and therefore bind DNA weakly, it can be argued that it is through the highly specific association with Max that the activities of these proteins are manifested. In general, Max interacting proteins have short half-lives and their biosynthesis is highly regulated. Max, on the other hand, is stable and constitutively expressed, suggesting that the regulation of the network is largely dependent on the abundance of the Max associated transcription factors [217, 218]. Our understanding of the Max network grew out of research on the *MYC* oncogene family. *MYC* was originally defined as an oncogene (v-*MYC*) transduced by a number of avian retroviruses capable of potently inducing neoplastic disease [219]. Subsequently c-*MYC*, the cellular homolog of v-*MYC*, was identified and eventually shown to be a member of a family of proto-oncogenes comprising c-*MYC*, *N-MYC*, and *L-MYC* (Fig. 13). These genes are considered proto-oncogenes in the sense that alterations in their structure and expression have been linked to a wide variety of human and other animal cancers [220-224]. The proteins (Myc) encoded by *MYC* family genes are predominantly localized in the cell nucleus, and their expression generally correlates with cell proliferation. When it was shown that the N-terminal region of Myc could function as a transcriptional activation domain [225] and that the C-terminal region possessed homology to bHLHZ proteins [226], it was widely assumed that Myc proteins would form homo- or heterodimers, bind DNA, and function as transcriptional activators. Because neither dimerization nor specific DNA binding could be readily demonstrated for Myc, except at high protein concentrations, a search for novel Myc interactors was initiated, leading to the identification of Max. Max was shown to interact specifically with all Myc family proteins, and the resulting heterocomplexes recognize the hexameric DNA sequence CACGTG (belonging to the larger class of sequences known as E-boxes, CANNTG) at concentrations at which binding by either partner alone is undetectable [227-229].

Importantly, Myc requires Max to activate transcription of genes containing E-box binding sites [215]. Furthermore, Myc has been shown to repress transcription at certain target promoters [230]. The transcription activation function of Myc is mediated at least in part by recruitment of a histone acetyltransferase (HAT) [231].

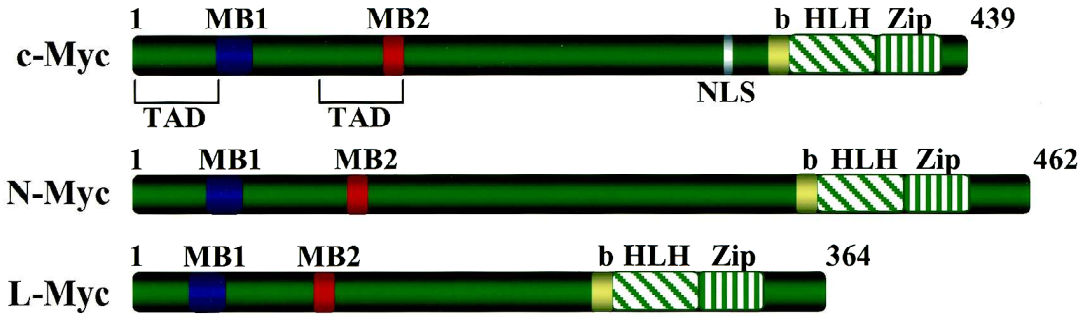


Figure 13. Structural domains of Myc oncoproteins. MB1 and MB2 domains are higly conserved within this family and are required for transactivation functions. MB2 is also required for the interaction with co- transcriptional activator TRAPP. TAD is a transactivation domain.

The fact that Max is expressed in the absence of Myc led to the question of whether Max might have additional dimerization partners. This prompted a search for new Max-interacting proteins by the use of expression cloning and two-hybrid screens. In this manner, two novel, but related, Max-interacting bHLHZ proteins were discovered, Mad1 and Mxi1, followed later by Mad3 and Mad4 [214]. These four proteins, considered to compose the Mad protein family, behave much like Myc in that they have only weak homodimerization and DNA-binding capacities but readily heterodimerize with Max and bind the E-box consensus sequence. However, in contrast to Myc, which activates transcription at promoters proximal to E-box sites, the Mad-Max heterodimers act as transcriptional repressors at the same binding sites. Each of the Mad proteins acts as a repressor by associating with the mSin3 corepressor complex [232]. Also, in contrast to Myc, expression of Mad family proteins appears closely linked to terminal differentiation [214]. Max interacts with at least two other bHLHZ proteins in addition to the Myc and Mad family members, Mnt (also called Rox) and Mga. Both of these proteins possess transcriptional activities which appear to be context dependent. Thus Mnt, similar to the Mad family proteins, recruits the mSin3 corepressor complex and represses transcription in some, but not all, cell types [233, 234]. Mga contains two functional DNA-binding domains, a bHLHZ region, which interacts with Max, and a Brachyury or T-box domain. Activation of transcription by Mga at T-box binding sites depends on binding of Max to the distal bHLHZ domain, suggesting that dimerization with Max displaces a repressor or induces a conformational change in Mga [214]. Although yet other Max-interacting proteins are likely to be identified, the basic outlines of the Max network are emerging. Max is a stable, ubiquitously expressed protein with little transcriptional activity of its own. The ability of Max to heterodimerize with several distinct groups of highly regulated proteins (outlined in Fig. 12) results in transcriptional activation

or repression directed at specific sets of target genes. Furthermore, the different complexes may have antagonistic properties whose functions play out at the level of chromatin structure. The involvement of these proteins in key biological events suggests that network function may be critical for growth and development. Indeed, the recent report that targeted deletion of Max results in very early embryonic lethality in mice underscores the importance of Max-dependent functions [235].

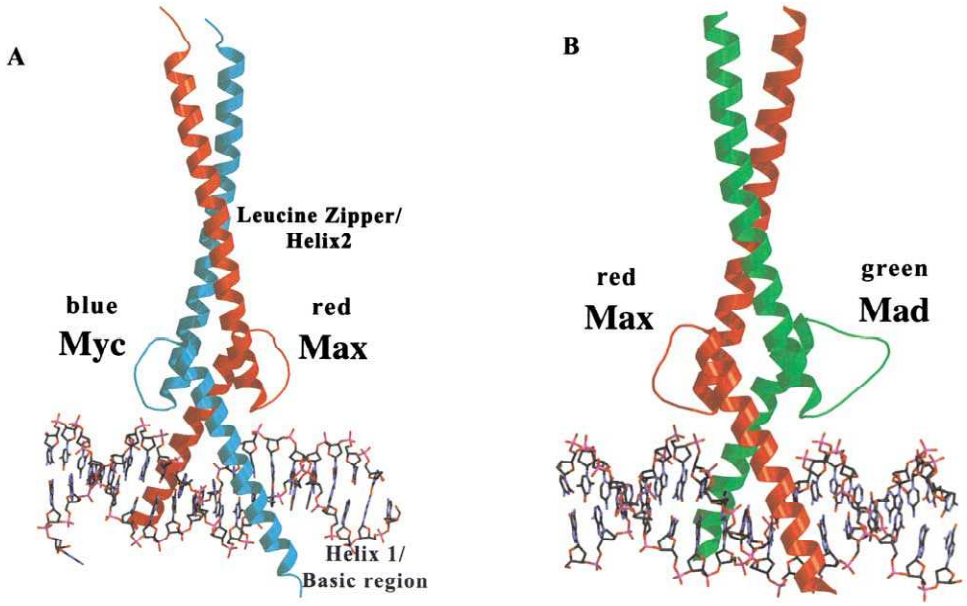


Figure 14. a) Structure of heterodimer Myc-Max bound to DNA; **b)** Structure of heterodimer Mad-Max bound to DNA.

BIOLOGICAL FUNCTIONS OF MYC

The intense scrutiny with which *MYC* has been studied over the last 15 years derives mainly from its apparent involvement in a wide range of cellular processes including proliferation, differentiation, and tumorigenesis. In the following sections we summarize the evidence for *MYC*'s role in both normal and abnormal cellular behavior, with special attention to the effects of deregulated expression of *MYC*.

INDUCTION BY MITOGENIC SIGNALS

One of the most compelling ideas about *MYC* is that it functions to drive proliferation in response to diverse signals. This notion arises from several broad lines of evidence which will be briefly reviewed here. First, *MYC* family genes are broadly expressed during embryogenesis, and targeted deletions of *c-MYC* or *N-MYC* genes in mice lead to lethality in midgestation embryos [236-242]. Second, there is a strong correlation between *MYC* expression and proliferation. This probably applies to all *MYC* family genes but has been most extensively documented for *c-MYC*. *MYC* expression is known to be induced in many cell types by a wide range of growth factors, cytokines, and mitogens [243-245]. The increase in *MYC* levels has been shown to occur through

both transcriptional and post-transcriptional mechanisms [246] and appears to occur as an immediate early response to most mitogenic factors, suggesting that the *MYC* regulatory region is a nexus for multiple growth signal response pathways. Regulation of translation initiation also occurs upon mitogenic stimulation [247]. For a number of receptors (including those for interleukin-2, macrophage colony-stimulating factor, Epo, epidermal growth factor, platelet-derived growth factor, and antigens), it can be argued that induction of *MYC* is a necessary, but probably not sufficient, component of the mitogenic response. Moreover, a failure to induce *MYC* in response to mitogenic signalling inhibits quiescent cells from entering the cycle. In contrast, ligands such as transforming growth factor beta and gamma-interferon, which in some settings act to inhibit proliferation, also cause rapid down-regulation of *c-MYC* expression [214]. Thus *MYC* expression strongly correlates with growth and proliferation.

MYC OVEREXPRESSION

The idea that Myc plays a critical role in the proliferative process is also consistent with results from experiments involving its ectopic expression in a variety of different cell types under a range of conditions. In these experiments, *MYC* expression is uncoupled from its normal physiological regulation; it is overexpressed and cannot be down-regulated. *MYC* overexpression in cycling cells has been reported to reduce requirements for growth factors, block exit from the cell cycle, accelerate cell division, and increase cell size [248-251]. In the absence of survival factors, c-Myc overexpression elicits a proliferative response but leads to apoptosis through a mechanism at least partly dependent on the Arf-Mdm2-p53 pathway [252-254]. Experiments with primary murine embryo fibroblasts (MEFs) demonstrate that loss of p53 or Arf greatly attenuates c-Myc-induced apoptosis and permits cells to survive crises and proliferate continuously in the absence of serum [253]. In some cell types, *MYC* requires coexpression of other genes (such as *RAS*, *JUN*, and *FOS*) in order to drive entry into S phase [214]. Myc levels rapidly diminish during the terminal differentiation of many cell types, and enforced expression of Myc inhibits or modulates terminal differentiation of myoblasts, erythroleukemia cells, adipocytes, B lymphoid cells, and myeloid cells among others. There is some evidence that Myc may not directly interfere with the programmed expression of differentiation genes but rather is incompatible with the cell cycle exit required for terminal differentiation [214]. However, not all differentiation events involve cell cycle arrest, and there are indications that Myc may play a role in advancing cells along pathways of epidermal and hematopoietic differentiation. Perhaps Myc is important for changes in cell growth and metabolism that are required for lineage commitment.

MYC DEREGULATION IN TUMOURS

The ability of overexpressed Myc to facilitate roliferation and inhibit terminal differentiation fits well with the fact that tumours of iverse origins contain genetic rearrangements involving *MYC* family genes. These rearrangements include retroviral transductions, amplifications, and chromosomal translocations, as well as viral insertions, and in general are thought to increase *MYC* expression levels and prevent *MYC* turnoff rather than alter the function of the Myc protein through mutation [220-224, 255]. Indeed, many of the genomic alterations in *MYC* result in increased *MYC* mRNA

levels through increased transcription initiation, decreased transcription attenuation, and augmented stability of the *MYC* messenger RNA [246]. However, although mutations in the Myc protein are relatively rare, they nonetheless do occur, particularly in retrovirus-transduced *MYC* genes and in translocated *MYC* genes [214]. It has been suggested that these mutations influence Myc's transactivation ability by altering binding to inhibitors such as the retinoblastoma protein (Rb) or p107 or by modulating the effects of serine and threonine phosphorylation required for full transcriptional activity [214]. Another possibility is that the transcriptional effects of mutations are secondary to alterations in Myc protein degradation. Myc family proteins have short half-lives, on the order of 20–30 min [256], and, although some instances of stabilization have been detected, it was reported earlier that in most tumour-associated Myc proteins, stability was not consistently affected [257, 258]. More recently, it has been discovered that Myc degradation is carried out through the ubiquitin-mediated proteosome pathway [259, 260] and that many tumour-related mutations in Myc result in significant stabilization of the protein [260]. Furthermore, activated Ras, an oncoprotein that collaborates with Myc in the transformation of primary cells, has also been reported to induce stabilization of Myc protein [261]. The molecular consequences of Myc stabilization are unknown, but the heightened stability must contribute to an overall increase in Myc protein levels and is likely to exacerbate the transcriptional effects of Myc. In this regard it is intriguing that the highly conserved region called Myc box II (see below), which is essential for transformation and at least some of Myc's transcriptional activities, has also been shown to regulate Myc protein turnover [260]. There is also evidence that c-Myc translation can be regulated by the switch from an apparently inefficient cap-dependent mechanism to a capindependent internal ribosome entry site within the 50 untranslated region [262]. This has been shown to occur during apoptosis as well as serum stimulation and has been suggested to also increase the rate of Myc translation during transformation [262]. The idea that the tumor-associated Myc mutations serve to activate Myc is attractive. However, a study systematically examining the effects of these mutations on Myc function in tissue culture cells has revealed that they have little if any effect on transformation, proliferation, apoptosis, or target gene expression [263]. Indeed the most common Myc mutation found in Burkitt's lymphomas, T58I, confers decreased transforming activity, with no evident change in apoptosis compared with wild-type Myc. These results may reflect important differences between in vitro transformation assays and in vivo tumorigenesis. Furthermore, the maintenance of apoptotic function in these Myc mutants may simply come from the ability of tumoUr cells to circumvent Myc-induced cell death by other mechanisms such as loss of p53 or Arf activities. Studies of Myc's role in oncogenesis indicate that augmented Myc levels can arise through transcriptional, post-transcriptional, and post-translational mechanisms. Several recent studies using murine models of epithelial and hematopoietic transformation have demonstrated that high *MYC* levels are continuously required to maintain the tumorigenic phenotype [264, 265]. Because, as described above, Myc overexpression in tissue culture cells can result in extended proliferation, it seems likely that similar effects occur in tumour cells and that secondary mutations in other genes cooperate with *MYC* to generate overt tumours. Another, not mutually exclusive role for Myc may be to induce genomic instability [264, 266, 267]. Although the mechanism for this has not been established, instability might generate cooperating mutations in other genes. A large number of genes that cooperate with *MYC* in mouse models of lymphomagenesis have been identified.

While in many cases the functions of these cooperating genes are unknown, recent work suggests that an important subset is likely to act by abrogating the apoptotic function of Myc. This notion had surfaced earlier when it was found that the antiapoptotic protein Bcl-2 inhibited Myc-induced apoptosis in tissue culture cells and also promoted lymphomagenesis in mice in collaboration with Myc [268, 269]. More recent experiments have demonstrated that in mice carrying a *c-MYC* transgene whose expression is directed to lymphoid cells (Eμ-*MYC* mice) [270] the *MYC*-overexpressing progenitor cells exhibit high rates of spontaneous apoptosis and contain an intact Arf-Mdm2-p53 checkpoint pathway [271]. However, lymphomas derived from these same mice display spontaneous inactivation of the p53 pathway through mutation or loss of p53 or Arf or via elevation of Mdm2 levels [271], nicely echoing the findings in MEFs [253] and in K562 cells, where Myc has also been shown to antagonize the effects of p53. As expected from these results, *MYC*-transgenic mice with hemizygous or null *ARF* alleles showgreatly accelerated lymphomagenesis [271], as do Eμ-*MYC* mice lacking p53 [272]. The importance of Arf and apoptosis in *myc*-induced lymphomagenesis was strikingly underscored by experiments demonstrating that the protein encoded by the cooperating oncogene *BMI1* is a member of the Polycomb class of repressors that acts to suppress expression from the *Ink4a* locus, which encodes Arf and p16INK4a [273]. Moreover, loss of one or both *bmi1* alleles inhibits lymphomagenesis in Eμ-*MYC* mice by increasing Arf-dependent apoptosis [274]. This work represents perhaps the clearest example of a mechanism through which Myc collaborates with other genes during tumour evolution. It is likely that other cooperating oncogenes will similarly act to evade the apoptosis inducing activity of deregulated *MYC*.

TRANSCRIPTION ACTIVATION AND THE Myc BOX II RIDDLE

When introduced into cells, Myc can activate transcription of synthetic reporter genes containing promoter proximal E-boxes in both yeast and mammalian cells [215, 216]. In addition, Myc stimulates natural E-box-containing promoters or sequences derived from putative Myc target genes [229, 275-277]. This transcriptional activity appears to require two regions of Myc: the C-terminal bHLHZdomain and the N-terminal transactivation domain comprising the first 143 amino acids [215, 216, 225]. The implication from these results is that Myc heterodimerizes with the ubiquitously expressed endogenous Max to permit sequence-specific DNA binding followed by Myc-dependent activation of transcription. This is consistent with many studies in which Myc bHLHZ mutations, leading to the loss of association with Max and/or decreased DNA binding, serve to abrogate Myc's transcriptional activation and biological activities. Further evidence supporting the necessity of Myc-Max interaction for transcriptional activation comes from experiments exchanging HLHZ domains of Myc and Max as well as altering the dimerization specificity of the zipper regions so that the mutant proteins dimerize with each other but not with their wild-type counterparts. These altered-specificity Myc and Max proteins fail to function on their own but are dependent on each other for stimulating transcription, proliferation, transformation, and apoptosis [278, 279]. Interestingly, artificial Myc homodimers generated through altered dimerization specificity-inducing mutations are deficient in biological function and transcriptional activation, suggesting that an interaction with Max is required for Myc-Max DNA binding or transcriptional activity. The dependence on Max for Myc function is consistent with other studies demonstrating that Myc-Max complexes can be detected in cells and that Max alone has little

transcriptional activity [217, 225]. Taken together, these experiments suggest a strong link between Myc-Max heterodimerization and transcriptional activation. While it seems likely that Myc's transactivation is crucial to its function, these studies offer no formal proof.

The region of Myc responsible for gene activation (the transactivation domain, or TAD) was initially defined by using fragments of c-Myc protein fused to the DNA binding domain of the yeast Gal4 protein [225]. These experiments mapped transcriptional activation to a segment lying between amino acids 1 and 143, which encompasses two regions, Myc box I (MBI; from approximately amino acids 45–63) and Myc box II (MBII; approximately amino acids 128–143), containing sequences highly conserved among the different Myc family proteins throughout evolution. An N-terminal region containing both MBI and MBII (amino acids 41–143) exhibits the highest transcriptional activity of any of the N-terminal Myc fusion proteins (Kato et al 1990). In this context, deletion of either MBI or MBII, as well as at least part of the sequence lying between them, diminishes transcriptional activation potential 10- or 50-fold, respectively. In biological assays, MBI deletion was found to attenuate only Myc transforming activity while an MBII deletion completely abolished it [280]. Although MBII is clearly required for Myc's transforming function, its role in transcription has been controversial. This is because deletion of MBII from the full-length c-Myc protein has little effect on activation in transient assays using either synthetic reporter genes or promoters derived from putative Myc target genes [230]. On the other hand, a careful study of the E-boxes in the promoter of α-prothymosin, a putative Myc target gene, shows that deletion of MBII eliminates Myc-induced activation at sites distal to the promoter while activation of more-proximal sites does not require MBII [281].

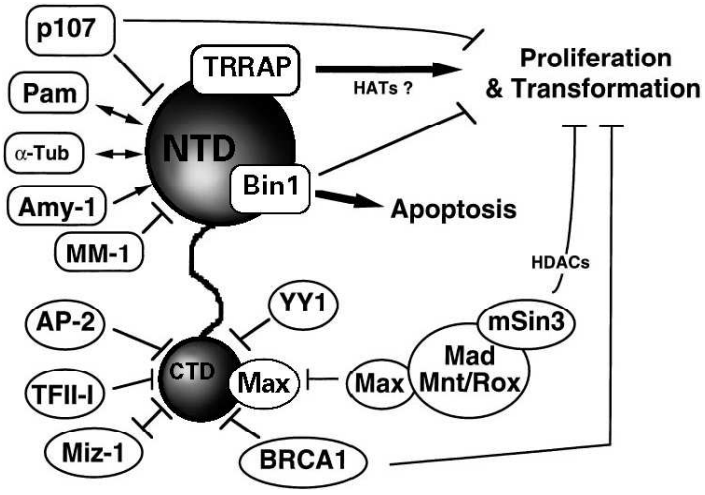


Figure 15. Factors which interact with Myc. CTD domain inlude "bHLHZ", and interacts with Max and other factors. NTD domain includes MB1 and MB2 regions involved in interactions with other proteins involved in transcriptional regulation, chromatin remodelling and apoptosis.

However, this detailed analysis has not been performed on the promoters of other target genes. Other work bearing on MBII and transactivation involves a naturally occurring variant of Myc, Myc-S (residues 101–439 of wild-type Myc), which lacks the majority of the transactivation domain but retains MBII. Myc-S has repression

activity but does not appear to function as a transactivator in transient assays [214]. Nonetheless, Myc-S can induce the expression of a number of endogenous Myc target genes. The correlation between the biological functions and the transcriptional activities of MBII is controversial because MBII has been shown to be important for Mycinduced repression. If MBII is not required for activation, then we might well conclude that cell transformation by Myc is more dependent on gene repression than on activation. Perhaps one problem is that assays using synthetic reporter genes, while capable of demonstrating the intrinsic activation or repression functions of transcription factors, may not faithfully reproduce the chromatin context characteristic of endogenous target genes. Indeed, the finding that MBII interacts with a coactivator complex with HAT activity argues for MBII's involvement in chromatin-dependent activation [231].

MECHANISMS OF Myc TRANSACTIVATION

The initial excitement generated by the iscoveries that Myc heterodimerizes with Max, binds DNA, and activates transcription oon gave way to the disappointing realization that Myc's ability to ransactivate both engineered reporters and putative endogenous target genes was relatively weak generally ranging from 3- to 10-fold transactivation when assayed in mammalian cells. In general, transactivation domains function by facilitating recruitment of the basal transcription machinery either directly or indirectly. In nearly all cases, TAD function involves interactions with other proteins. Although the N-terminal Myc TAD has been shown to associate with a number of proteins, few provide obvious clues to the mechanism of Myc-induced activation. A striking exception has been the recent identification of a novel nuclear cofactor called TRRAP as a Myc-binding protein. TRRAP was identified by using Myc-TAD as an affinity reagent to isolate interacting proteins and was shown to require MBII for binding [282]. The TRRAP sequence is homologous to the ATM/PI-3 kinase family, although amino acid changes in the active-site region suggest that TRRAP is unlikely to possess kinase activity [282]. Concomitantly, studies of the yeast *Saccharomyces cerevisiae* identified a protein highly related to TRRAP as a component of the SAGA complex, a molecular assembly containing the HAT GCN5 and other components which facilitate TBP positioning [283, 284].

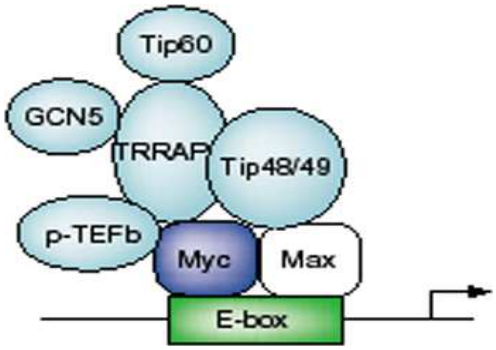


Figure 16. Myc regulates chromatin structure in close proximity to E-Box sequenze. MBII domain determines the recruitment of TRRAP and GCN5.

A mammalian counterpart of SAGA has been identified [285]. While it is still unclear whether this entire complex is associated with the Myc TAD, a recent report indicates that HAT activity coimmunoprecipitates with Myc protein both *in vitro* and *in vivo* [231]. The recruitment of the TRRAP-GCN5 complex places Myc, and specifical ly the MBII region of Myc, among the group of transcriptional activators. When considered with earlier work indicating that Mad-Max and Mnt-Max dimers repress transcription through recruitment of a corepressor complex containing histone deacetylase (HDAC) activity [286], these findings produce a satisfyingly symmetrical view of Myc and Mad functions and immediately suggest that Myc and Mad antagonism stems from the opposing enzymatic activities directed toward histone modification. Although many of the Mad proteins are induced during terminal differentiation, when Myc is downregulated, there is also evidence of Mad family and Mnt expression in proliferating and quiescent cells. One possible explanation for this is that the Myc-Max complex exhibits weak transactivation activity because its major role is not to fully activate targets but rather to augment the accessibility of regions of chromatin that have closed through the action of Mad- or Mnt-associated deacetylases. The region of chromatin opened through Myc would permit subsequent binding and activation by constitutive transcription factors (such as USF). This view of Myc-Max as a derepressor has received some support from recent work on the cyclin D2 promoter. Induction of a conditional Myc can activate expression of the endogenous D2 gene, although Myc is inactive in transient assays with the D2 promoter. When Mad-Max is used to repress the promoter, then Myc can induce its expression [287]. Interestingly, induction of cyclin D2 mRNA can also be achieved by treatment of cells with a HDAC inhibitor. This suggests that Myc may function most efficiently in a context of repression. Myc has also been linked to an SWI/SNF-like ATP-dependent chromatin remodelling complex. A recent report showed that Myc can interact in vivo with the SNF5 homolog INI1 through Myc's bHLHZ domain and that putative dominant interfering forms of INI1 and BRG1/hSNF2 can block Myc transactivation [288].
Because INI1 is a strong candidate tumor suppressor [289], its potential role as a positive mediator of Myc function is somewhat puzzling. A major unanswered question is whether INI1 acts to displace Max from the HLHZ region or forms a ternary complex with Myc and Max. Perhaps binding of the INI1 complex to Myc alters Myc's target specificity or transcriptional activity. In any event, it is intriguing to imagine that Myc recruits both histone-modifying and chromatin-remodeling activities. A number of other interesting proteins have been identified as being capable of associating with Myc and possibly influencing activation (or repression). For most of these proteins, functional information is limited, and it still remains to be determined whether interactions occur when the partners are expressed at physiological levels.

TRANSCRIPTIONAL REPRESSION BY Myc

For a number of years it has been observed that high levels of Myc expression in transformed cell lines correlate with down-regulation of specific mRNAs. These mRNAs include those encoding cell surface proteins such as the class I HLA molecules in melanoma cells, the $\alpha 3\ \beta 1$ integrin in neuroblastomas, and the LFA-1 (αL β2 integrin) cell adhesion protein in transformed B cells as well as H-ferritin [214, 289] . More recently a DNA element required for Myc-mediated repression has been demonstrated to lie within the promoters of repressed target genes, indicating that

Myc repression is likely mediated at the transcriptional level [230]. These studies have raised two major questions: first, what is the mechanism of Myc repression and, second, what roles do repression and activation play in Myc's biological functions? Do repression and activation cooperate to facilitate Myc function, and are they antagonistic or separable independent activities? The question of mechanism again leads to the highly conserved MBII region. MBII is required for repression of the growth arrest gene *GAS1* and for the down-regulation of the C/EBPα and the albumin promoter in transient assays [230, 290]. However, the region of Myc spanning residues 96–106 appears to be required for down-regulation of the cyclin D1 mRNA. Taken at face value, these studies indicate that there are at least two regions involved in repression and that these regions are contained within the transcriptional activation domain. Evidence suggesting that Myc repression may require both recognition of a specific DNA sequence and interaction with specific proteins has accumulated. A number of Myc-repressed targets contain a subclass of initiator elements (INRs; consensus, YYCAYYYYY, where Y is a pyrimidine base) which are usually, but not invariably, present at TATA-less promoters. Other Myc-repressed genes, such as *GADD45*, do not contain INR sequences; rather, repression appears to be mediated by a GC-rich region that is potentially recognized by WT1 and p53 [291]. INR elements are recognized by TFIID as well as a number of regulatory proteins, such as the transcription initiation factor TFII-I, YY-1, and the POZ domain protein Miz-1. Interestingly, the last three proteins have been reported to associate with the bHLHZ region of Myc [214]. While there has been little follow-up on the initial reports involving Myc interaction with TFII-I, the association of Miz-1 with Myc has been recently confirmed and shown to promote stabilization of Myc by inhibiting its ubiquitin-dependent degradation [260]. Perhaps a stable Myc-Miz1 interaction blocks the ability of Miz-1 to initiate transcription at INR-containing and other promoters [292]. Similarly, high Myc levels in the cell are thought to sequester YY-1 and prevent transcription of one of its target genes, although another report suggests that the effects of YY-1 on Myc are indirect [214]. There is also evidence that a naturally occurring Max protein variant (dMax) lacking a basic region and helix 1 can interact with c-Myc to block its transcriptional activation function but promote its repression activity [293]. Perhaps the common theme underlying Myc repression is a loss of Myc E-box binding function, for example by displacement of Max by other bHLHZ binding proteins, permitting Myc to associate with and sequester positively acting transcription factors (e.g. Miz-1). Here the role of MBII in recruiting TRRAP-HAT might involve not acetylation of chromatin at E-box sites but rather an inhibitory acetylation of the sequestered transcription factors. Indeed, there are several reports of acetylation altering protein specificity and activity [294].

Myc represses also the p21 promoter, which encodes for the cyclin-dependent kinase inhibitor, through the short GC-rich region just upstream of the transcription start site, although Myc does not appear bind directly its promoter. c-Myc does not need to heterodimerize with Max for repression of p21 promoter. this proximal region of the p21 promoter lacks Myc-canonical binding sites, but contains multiplr Sp1-binding sites and a potential Inr element. c-Myc mediated p21 repression is independent of histone deacetylase activity [295] (Fig. 17).

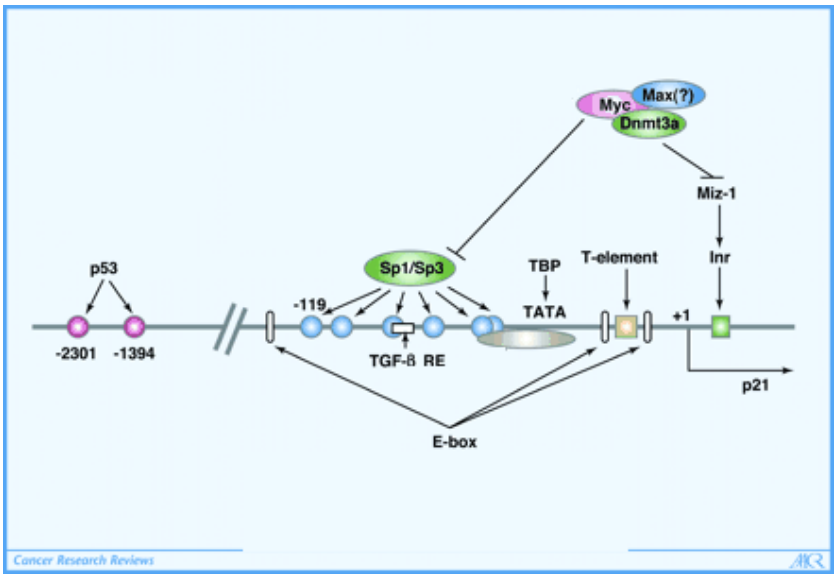


Figure 17. Multiple factors employ distinct mechanisms to repress the p21 promoter. Myc exerts their action through the proximal promoter region. Myc oncoproteins tend to interfere with positive regulators of p21 transcription, such as p21 and Miz1.

What is the relative importance of Myc's activation and repression functions? Unfortunately, an unequivocal answer to this question is not available. Recent data from DNA microarray experiments attempting to determine the effects of Myc overexpression on global gene expression profiles have demonstrated that multiple genes are both activated and repressed by Myc, with the majority being activated [214]. However, the relative importance of activated and repressed genes cannot be accurately assessed at this point. Nonetheless, it seems likely that both activation and repression are required for Myc biological function. Only when we learn more about the molecular details of Myc's transcriptional functions will we be able to generate mutations permitting us to distinguish the consequences of activation vs repression.

CELL CULTURES

Human neuroblastoma Tet21/N, SHEP cells were grown in DMEM containing 10% FBS and 50μg/ml gentamycin. Human neuroblastoma SH-SY-5Y, LAN-1 and LAN-5 were grown in RPMI medium 1640 containing 20% FBS and 50 μg/ml gentamycin. HL60, P493, Kazumi-4,K562, KG1A cells were grown in RPMI medium 1640 containing 10% heat-inactivated FBS and 50 μg/ml gentamycin.

RNA EXTRACTION

The step by step protocol is described for cultured cells grown in two 100-mm dishes, containing 1-1,5 x 107 cells per dish. Remove the medium and add slowly 1ml of PBS1X. wash and remove. Harvest the cells using trypsin treatment and when the cells detach from the culture dish, add 1 volume of fresh medium and transfer the sunspension to a tube. Centrifuge for 5 minutes at 1000 rpm, then remove the supernatant. Add 1-1,5 ml of TriReagent (Sigma). Pipet gently up and down and incubate for 5 minutes at room temperature. Add 300 μl of chloroform and vortex for 10 seconds. Incubate 5-10 minutes at room temperature. Centrifuge fo 5 minutes at 12000rpm at 4°C. transfer acqueous phase in a new tube an add 750 μl of isopropyl alcohol. Mix gently and incubate for 5-10 minutes at room temperature. Centrifuge at 12000rpm for 10 minutes at 4°C. remove the supernatant and wash the pellet with 1,5 ml EtOH 75% treated with DEPC and centrifuge at 12000 rpm for 5 minutes at 4°C. remove the supernatant and dry the pellet. Then, resuspend the pellet in 30-50 μl of DEPC-treated water and heat the sample at 55°C for 10 minutes.

THERMOSCRIPT RT-PCR SYSTEM

The ThermoScript RT-PCR was designed for the sensitive and reproducible detection and analysis of a RNA molecules in a two-step process. ThermoScript RT, an avian reverse transcriptase with reduced RNase H activity, was engineered to have higher thermal stability, produces higher yields of cDNA, and produce full-length cDNA. cDNA synthesis was performed using total RNA with oligo(dT).
In a 0,2-ml tube, combine primer (oligo(dT)), 2μg total RNA and dNTP 10mM mix, adjusting volume to 12 μl with DEPC-treated water. Denature RNA and primers by incubating at 65°C for 5 min and then place on ice. Vortex the 5X cDNA Synthesis buffer for 5 sec just prior to use. Prepare a master reaction mix on ice, with 5X synthesis buffer, 0,1M DTT, RNaseOUT (40U/ μl), DEPC-treated water and ThermoScript RT (15units/ μl). vortex this mix gently. Pipet 8 μl of master reaction mix into each reaction tube on ice. Transfer the sample to a thermal cycler preheated to the appropriate cDNA synthesis temperature and incubate for 100 min at 50°C. terminate the reaction by incubating at 85°C for 5 min. Add 1 μl of RNase H and incubate at 37°C for 20min. Add 80 μl of MQ-water for each reaction and store at -20°C or use for qPCR immediately. Use only 2-5 μl of the cDNA synthesis reaction for qPCR.

SYBR GREENER qPCR

SYBR GreenER qPCR SuperMix (Invitrogen) for ICycler is a ready to use cocktail containing all components, except primers and template, for real-time quantitative PCR (qPCR) on ICycler BioRad real time instruments that support normalization

with Fluoresceina Reference Dye at final concentration of 500nM. It combines a chemically modified "hot-start" version of *Taq*DNA polymerase with integrated uracil DNA glycosilase (UDG) carryover prevention technology and a novel fluorescent dye to deliver excellent sensitivity in the quantification of target sequences, with a linear dose response over a wide range of target concentrations. SYBR GreenER qPCR SuperMix for ICycler was supplied at a 2X concentration and contains hot-start *Taq*DNA polymerase, SYBR GrenER fluorescent dye, 1 μM Fluorescein Reference Dye, $MgCl_2$, dNTPs (with dUTP instead of dTTp), UDG, and stabilizers. The SuperMix formulation can quantify fewer than 10 copies of a target gene, has a broad dynamic range, and is compatible with melting curve analysis. The *Taq*DNA polymerase provided in the SuperMix has been chemically modified to block polymerase activity at ambient temperatures, allowing room-temperature set up and long term storage at 4°C. Activity is restored after a 10-minutes incubation in PCR cycling, providing an automatic hot start for increased sensitivity, specificity and yield. UDG and dUTP in the SuperMix prevent the reamplification of carryover PCR products between reactions. dUTP ensures that any amplified DNA will contain uracil, while UDG removes uracil residues from single or double-stranded DNA. A UDG incubation step before PCR cycling destroys any contaminating dU-containing product from previous reactions. UDG is then inactivated by the high temperatures during normal PCR cycling, thereby allowing the amplification of genuine target sequences. Fluorescein is included at a final concentration of 500nM to normalize the fluorescent signal on instruments that are compatible with this option. Fluorescein can ajust for non-PCR-related fluctuations in fluorescence between reactions and provides a stable baseline in multiplex reactions. Program real time instrument for PCR reaction as shown following: 50°C for 2 minutes hold (UDG incubation), 95°C for 10 minutes hold (UDG inactivation and DNA polymerase activation), 40 cycles of: 95°C for 15 seconds and 60°C for 60 seconds. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well, and then the unique reaction components (e.g. template, forward and reverse primers at 200nM final concentration). Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate, centrifuge briefly and place reactions in a pre-heated real-time instrument programmed as described above.

MTT ASSAY

MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular proliferation (cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxide or a solution of the detergent sodium dodecyl sulfate in dilute hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

MTT stock solution: 5mg/ml (Promega) in RPMI-1640 without phenol red. This solution is filtered through a 0,2 μm filter and stored at 2-8°C.

MTT working solution: 1:10 diluition of the 5mg/ml stock

Wash cultured cells with warm RPMI-1640 withot phenol red. Prepare MTT working solution. Add MTT working solution into wells being assayed, for example 1 ml for each well of 12-well plate. Incubate at 37°C for 30min to 3hrs (this time depends on cell density and cell type). At the end of the incubation period, the medium can be moved if working with attached cells. the converted dye is solubilized with 1ml acidic isopropanol (0,04M HCl in absolute isopropanol). Pipette up and down several times to make sure the converted dye dissolves completely. Transfer the dye solution with the cells into a 1,5ml eppendorf tube and centrifuge at 13000 rpm for 2 min. transfer the supernatant into a new eppendorf tube. Absorbance of the converted dye is measured at a wavelenght of 570nm with background subtraction at 650nm. For the measurement, use Beckman DU-600 Spectophotometer and disposable plastic.

MULTI-DRUG EFFLUX ASSAY

The Multidrug Resistance Direct Dye Efflux Assay Kit (Chemicon) includes two of the best characterized and most commonly used multidrug resistance ABC transporter substrates, DiOC2(3) and rhodamine 123. The kit enables researchers to directly assess the functional activity of the MDR1, MRP1 and BCRP membrane pumps in living cells under physiologic conditions by directly measuring the relative fluorescence of cell populations that actively extrude fluorescent multidrug resistance transport substrates.

Cold Efflux Buffer – In a sterile field, mix the contents of the 200 ml sterile 5x RPMI-1640 bottle (200 ml) and 800 ml sterile water (tissue culture grade) in a sterile 1 liter bottle. With a sterile pipet, add 34.5 ml of the sterile 30% BSA to obtain sterile efflux buffer. If desired, to maintain sterility, add 1 ml of Gentamicin Solution, 1000x, and mix. Chill on ice for at least 2 h before using in the assay. Open the container only in a sterile environment. Store unused portion at 4ºC for up to 12 months. Discard if contamination is evident. Approximately 10 ml Efflux Buffer total is required for each test.
Warmed Efflux Buffer – Remove an aliquot of Cold Efflux Buffer and warm to 37ºC at least one hour before use in the assay; keep in water bath at 37°C until use.
DiOC2(3) Loading Buffer – On the day of the assay, dilute DiOC2(3) Solution 1:1000 in the desired amount of Cold Efflux Buffer. A volume of 250 µl per test is required. Invert 5-10 times to mix. Keep on ice until adding to cells. Protect solutions containing DiOC2(3) from prolonged exposure to light.
Rhodamine 123 Loading Buffer – On the day of the assay, dilute Rhodamine 123 Solution 1:100 in the desired amount Cold Efflux Buffer. A volume of 250 µl per test is required. Invert 5-10 times to mix. Keep on ice until adding to cells. Protect solutions containing Rhodamine 123 from prolonged exposure to light.
Note: *The user has the choice of using either DiOC2(3) or Rhodamine 123, or both as two separate experimental points, as an efflux probe in a given experiment, depending on the application of the user.*
Warmed Efflux Buffer containing 22 µM Vinblastine – On the day of the assay, dilute Vinblastine Solution 1:1000 in Warmed Efflux Buffer. For tests to assess vinblastine inhibition of dye efflux, a volume of 1 ml per test is required. Keep at

37ºC until use. Warmed Efflux Buffer containing DMSO – On the day of the assay, dilute DMSO 1:1000 in Warmed Efflux Buffer. For tests to assess dye efflux in the absence of vinblastine, a volume of 1 ml per test is required. Keep at 37ºC until use Propidium Iodide Buffer – Dilute Propidium Iodide Stock Solution 1:50 in Cold Efflux Buffer before use in flow cytometry. A volume of 0.5 ml per test is required. Protect solutions of Propidium Iodide from prolonged exposure to light. (Optional) Cold efflux buffer or other antibody staining buffer containing 0.01% sodium azide. Needed if staining with an antibody will be performed after the efflux assay.

Amounts of reagents required per test:

	treatment			
item	**4°C**	**37°C + DMSO**	**37°C + vinblastine**	**37°C + experimental compound**
cells	2.5 x 10^5 or more	2.5 x 10^5 or more	2.5 x 10^5 or more	2.5 x 10^5 or more
$DiOC_2(3)$ or Rhodamine 123 Loading Buffer	0.25 ml	0.25 ml	0.25 ml	0.25 ml
Cold Efflux Buffer (for washing)	7.5 ml	7.5 ml	7.5 ml	7.5 ml
Cold Efflux Buffer (for efflux step)	1 ml			
Warmed Efflux Buffer + DMSO		1 ml		
Warmed Efflux Buffer + vinblastine			1 ml	
Warmed Efflux Buffer + experimental compound				1 ml
Propidium Iodide Buffer	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Assay Protocol

Note: *Because membrane transport mediated by MDR1, MRP1 and BCRP is a complex process that is highly dependent on multiple factors, such as physiological conditions of the target cell population, intracellular ATP status, the degree of expression of MDR1, MRP1 and BCRP, and fluorescent dye concentration, some parameters may need to be adjusted for each experimental series. At the same time, it is essential that all temperature conditions and media formulations strictly correspond to the underlying protocol.*

Grow cell line of choice in its appropriate medium. The cells should be kept in media lacking multidrug resistance substrates for at least one week (selective drugs may interfere with dye efflux). Antimicrobial agents appear not to interfere with multidrug resistance ABC transporter function and may be included in the media. Media should be replaced one day before the assay. Approximately 2.5 x 100000 cells are required for each test**.** Adherent cells should be dislodged from plates by standard methods, and used in the assay in suspension. Count cells. Calculate the total number of cells required for the number of tests to be performed, and take volume necessary to get

the desired number of cells. Centrifuge cells at 200 x g for 5 min. Discard the supernatant and retain the cell pellet. Resuspend the cell pellet at 1 x 106 cells/ml in cold DiOC2(3) or Rhodamine 123 Loading Buffer.

Note: *At this step, cells intended to be used in separate tests can be loaded with the fluorescent dye of choice in one tube. Minimize light exposure of dye-containing samples by handling quickly when in ambient light, and performing incubations in containers that offer protection from light.*

For loading with DiOC2(3), incubate for 15 min on ice. For loading with Rhodamine 123, incubate for 30 min to 2 h on ice. Centrifuge cells at 200 x g for 5 min. Remove supernatant. Resuspend pellet in 2.5 ml Cold Efflux Buffer per 106 cells. Repeat previous step. At this point, distribute resuspended cells into different tubes for each different treatment. For an initial confirmation of specificity of efflux, use 3 tubes, each containing 625 µl (2.5 x 105 cells).

Note: *An initial characterization of the time course of dye efflux by each individual cell type is strongly recommended. For time course experiments, the quantity of cells undergoing the same treatment can initially be put in the same tube. For example, for* ***n*** *time points, put* ***n*** *x 2.5 x 100000 cells(****n*** *x 625* µl *at the cell density given in step 8) into one tube for each treatment.*

Centrifuge cells at 200 x g for 5 min. Remove supernatant. Resuspend cells in the following media, at 1 ml per test containing 2.5 x 100000 cells:

A. 37ºC-Warmed Efflux Buffer containing DMSO
B. 37ºC-Warmed Efflux Buffer containing vinblastine
C. Ice-Cold Efflux Buffer

Immediately transfer tubes **A** and **B** to a 37ºC water bath. Do not perform incubations in an incubator, which does not have adequate temperature control. Keep tube **C** on ice. Incubate for the desired time period.

Note: *For time course experiments, remove 1 ml aliquots from the incubation tube, add to Cold Efflux Buffer, and immediately return the incubation tube to its proper incubation temperature to continue the time course. Cell types with high levels of MDR1 expression (e.g. KB-8-5-11 or KBV-1 cell lines) tend to efflux dyes within 15 min. Cell types expressing lower amounts or endogenous MDR1 (e.g. normal lymphocytes or hematopoietic stem cells) will require 30 min to 3 h to efflux dyes.*

Add 5 ml Cold Efflux Buffer per test, and immediately put the tube on ice.

Note: *Low temperatures stop the efflux reaction. For time course experiments, the earlier time points may be kept on ice at this point until all of the samples are collected.*

Centrifuge at 200 x g for 5 min in a refrigerated centrifuge at 4ºC. Remove supernatant. Resuspend cells in 1 ml per test (2.5 x 105 cells) Cold Efflux Buffer. Centrifuge at 200 x g for 5 min in a refrigerated centrifuge at 4ºC. Remove

supernatant.(Optional) If antibody staining of cells subjected to the efflux assay is desired, use a PE-conjugated antibody and perform the staining using ice-cold buffers after the efflux phase is completed For analysis by flow cytometry, resuspend cells in 0.5 ml per test Cold Propidium Iodide Buffer. For analysis in a fluorescence plate reader, resuspend cells in 0.25 ml per test Cold Efflux Buffer. Maintain on ice until analysis by flow cytometry or fluorometry. Cells may be kept on ice for several hours. Longer times are not recommended, as prolonged exposure to the dyes and vinblastine can be toxic to the cells. Analyze by flow cytometry, with DiOC2(3) and rhodamine 123 on FL1, PE (if employed in step 18) on FL2, and PI on FL3. Collect 2500-10,000 events. Alternatively, cell suspensions can be dispensed into the wells of a blackwalled 96-well plate and measured in a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

ChIP- CHROMATIN IMMUNOPRECIPITATION

The step by step protocol is described for cultured cells grown in two 100-mm dishes, containing 1-1,5 x 107 cells per dish. Two 100-mm dishes are used for each immunoprecipitation. In the specific case the protocol is intended for human neuroblastoma cells growing adhesively. Minor adjustments have to be introduced for other cell types especially for those growing in suspension. Based on our experience, one of the most critical steps in performing ChIP regards the conditions of chromatin fragmentation, which need to be empirically set up for each cell types employed.

In each plate add 270 µl formaldehyde from a 37% stock solution and mix immediately. Incubate samples on a platform shaker for 10 minutes at room temperature. In each plate add 500 ml glycine from a 2,5 M stock solution and mix immediately. Incubate on a platform shaker for 10 minutes at room temperature. Transfer the plates in ice and remove the medium. Harvest the cells with a scraper and then centrifuge at 1500 rpm for 4 minutes in cold centrifuge, then keep samples on ice. Remove the supernatant and wash pellet 3 times with 10 ml ice-cold PBS1X/ 1 mM PMSF. After each washing centrifuge at 1500 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 500 µl ice-cold Cell Lysis Buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Centrifuge at 3000 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 600 µl ice-cold RIPA buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Sonication of crosslinked cells is performed in two distinct steps. First, cells are sonicated with a Branson Sonifier 2 times for 15 seconds at 40% setting. Next, cell samples are further sonicated with the Diogene Bioruptor for 20 minutes at high potency in a tank filled with ice/water in order to keep cell samples at low temperature during sonication. Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and pre-clear lysate by incubating it with 50 µl of Immobilized Protein A (Pierce) for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA, and add 5 µg of specific antibody. Rotate the sample O/N in the cold room. Add 50 µl of Immobilized Protein A (Pierce) and incubate by constant rotation for 30 minutes at room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation fro 3 minutes at room temperature and the centrifuge at 4000 rpm for 2 minutes at room temperature. Wash 4 times with 1 ml Ripa Buffer.

Wash 4 times with 1 ml Washing Buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer to the beads. Add 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 50 µl Proteinase K Buffer 5X and 6 µl Proteinase K (19 mg/ml). Then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (250 µl) to a new tube.
Extract once with phenol/chlorophorm/isoamylalcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 100 µl TE buffer to the remaining phenol/chlorophorm fraction and re-extract DNA. Recover the aqueous phase and add it to the previous one. Extract once with chlorophorm/iso-amyl-alcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 1 µl glycogen (Glycogen is 20 mg/ ml stock solution), 10 µg Salmon Sperm, 1/10 volumes Na-acetate 3M pH 5.2, and 2.5 volumes of cold ethanol100% Vortex and precipitate at -80°C for 40 minutes. Centrifuge at 14000 rpm for 30 minutes at 4°C. Remove the supernatant and wash pellet with 200 µl EtOH 70%. Resuspend IP-DNA and INPUT samples in 50-100 µl 10 mM TrisHCl pH 8. Use 2-4 µl of IP-DNA for Real Time PCR analysis.

Cell Lysis Buffer:	**RIPA Buffer**	**Washing buffer**
5 mM PIPES pH 8	150mM NaCl	100mMTrisHCl pH 8
85 mM KCl	1% NP40	500mM LiCl
0,5% NP40	0,5% NaDoc	1% NP40
1 mM PMSF	0,1% SDS	1% NaDoc
Protease inhibitor cocktail (Roche)	50 mM TrisHCl pH 8	
	1 mM PMSF	
	Protease inhibitor cocktail (Roche)	

DUAL-STEP CHROMATIN IMMUNOPRECIPITATION

The step by step protocol is described for cultured cells grown in two 100-mm dishes, containing 1-1,5 x 107 cells per dish. Two 100-mm dishes are used for each immunoprecipitation. In the specific case the protocol is intended for human neuroblastoma cells growing adhesively. Minor adjustments have to be introduced for other cell types especially for those growing in suspension. Based on our experience, one of the most critical steps in performing ChIP regards the conditions of chromatin fragmentation, which need to be empirically set up for each cell types employed.

Remove medium and add 2 ml PBS 1X/ 1 mM PMSF to each plate and scrape cells at room temperature. Pool together the cells from two plates and centrifuge at 1500 rpm for 5 minutes at room temperature. Wash cell pellet with 20 ml PBS1X/ 1 mM PMSF at room temperature and centrifuge at 1500 rpm for 5 minutes. Repeat this step 3 times. Resuspend pellet in 20 ml PBS1X/ 1 mM PMSF. Add disuccinimidyl glutarate (DSG) to a final concentration of 2mM and mix immediately. DSG is prepared as a 0.5 M stock solution in DMSO. (Note1) Incubate for 45 minutes at room temperature on a rotating wheel at medium speed (8-10 rpm). At the end of fixation, centrifuge the sample at 1500 rpm for 10 minutes at room temperature. Wash cell pellet with 20 ml PBS1X/ 1 mM PMSF at room temperature and centrifuge at 1500 rpm for 5 minutes. Repeat this step 3 times. Resuspend pellet in 20 ml PBS1X/ 1 mM PMSF. Add 540 µl formaldehyde from a 37% stock solution and mix immediately. Incubate samples on a rotating wheel for 15 minutes at room temperature. Add 1 ml glycine from a 2,5 M stock solution and mix immediately.

Incubate on a rotating wheel for 10 minutes at room temperature. Centrifuge samples at 1500 rpm for 4 minutes in cold centrifuge, then keep samples on ice. Remove the supernatant and wash pellet 3 times with 10 ml ice-cold PBS1X/ 1 mM PMSF. After each washing centrifuge at 1500 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 500 µl ice-cold Cell Lysis Buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Centrifuge at 3000 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 600 µl ice-cold RIPA buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Sonication of crosslinked cells is performed in two distinct steps. First, cells are sonicated with a Branson Sonifier 2 times for 30 seconds at 40% setting. Next, cell samples are further sonicated with the Diogene Bioruptor for 20 minutes at high potency in a tank filled with ice/water in order to keep cell samples at low temperature during sonication. (Note 3) Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and preclear lysate by incubating it with 50 µl of Immobilized Protein A (Pierce) for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA, and add 5 µg of specific antibody. Rotate the sample O/N in the cold room. Add 50 µl of Immobilized Protein A (Pierce) and incubate by constant rotation for 30 minutes at room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation fro 3 minutes at room temperature and the centrifuge at 4000 rpm for 2 minutes at room temperature. Wash 4 times with 1 ml Ripa Buffer. Wash 4 times with 1 ml Washing Buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer to the beads. Add 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 50 µl Proteinase K Buffer 5X and 6 µl Proteinase K (19 mg/ml). Then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (250 µl) to a new tube.
Extract once with phenol/chlorophorm/isoamylalcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 100 µl TE buffer to the remaining phenol/chlorophorm fraction and re-extract DNA. Recover the aqueous phase and add it to the previous one. Extract once with chlorophorm/iso-amyl-alcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 1 µl glycogen (Glycogen is 20 mg/ ml stock solution), 10 µg Salmon Sperm, 1/10 volumes Na-acetate 3M pH 5.2, and 2.5 volumes of cold ethanol100% Vortex and precipitate at -80°C for 40 minutes. Centrifuge at 14000 rpm for 30 minutes at 4°C. Remove the supernatant and wash pellet with 200 µl EtOH 70%. Resuspend IP-DNA and INPUT samples in 50-100 µl 10 mM TrisHCl pH 8 Use 2-4 µl of IP-DNA for Real Time PCR analysis.

Notes

1).We have tested several crosslinking agents including DSG (disuccinimdyl glutarate), EGS [ethylene glycol bis(succinimidylsuccinate], DMA (dimethyl adipimidate) and DSS (disuccinidimyl suberate). In our conditions, DSG was the one that worked best, although we also obtained good results with EGS.
2) Sometimes, insoluble aggregates form when DSG is added to cells resuspended in PBS 1X . However, this seems not to preclude the efficiency of the crosslinking reaction.
3) Through this procedure we could efficiently fragment chromatin in a range between 500 and 200 bp. As stated above, this is a critical step that must be

empirically set up for each cell line tested. For example, HL-60 cells that grow in suspension, are sonicated with a Branson Sonifier 4 times for 30 seconds at 40% setting and subsequently with the Biogene Bioruptor at a full power for 30 minutes. This procedure allows fragmentation of HL-60 chromatin to a size range of 1000-500 bp.

LUCIFERASE ASSAY

The Dual-Luciferase® Reporter (DLR.) Assay System (Promega) provides an efficient means of performing dual-reporter assays. In the DLR. Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a stabilized signal from the *Renilla* luciferase, which decays slowly over the course of the measurement. In the DLR. Assay System, both reporters yield linear assays with subattomole sensitivities and no endogenous activity of either reporter in the experimental host cells. Furthermore, the integrated format of the DLR. Assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions.

Note: The LAR II, Stop & Glo® Reagent and samples should be at ambient temperature prior to performing the Dual-Luciferase® Assay. Prior to beginning this protocol, verify that the LAR II and the Stop & Glo® Reagent have been warmed to room temperature.

The assays for firefly luciferase activity and *Renilla* luciferase activity are performed sequentially using one reaction tube. The following protocol is designed for use with a manual luminometer or a luminometer fitted with one reagent injector.
Predispense 100µl of LAR II into the appropriate number of luminometer tubes to complete the desired number of DLR. Assays. Program the luminometer to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay. Carefully transfer up to 20µl of cell lysate into the luminometer tube containing LAR II; mix by pipetting 2 or 3 times. **Do not vortex**. Place the tube in the luminometer and initiate reading.

Note: We do not recommend vortexing the solution at Step 3. Vortexing may coat the sides of the tube with a microfilm of luminescent solution, which can escape mixing with the subsequently added volume of Stop & Glo® Reagent. This is of particular concern if Stop & Glo® Reagent is delivered into the tube by automatic injection.

If using a manual luminometer, remove the sample tube from the luminometer, add 100µl of Stop & Glo® Reagent and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.Discard the reaction tube, and proceed to the next DLR. Assay.

CO-IMMUNOPRECIPITATION AND GST-PULL DOWN ASSAYS

The interaction between different proteins is assessed by immunoprecipitation and Western blotting. Cells are washed two times in PBS 1X+ PMSF (0,1%) and lysed in the following buffer for isolation of nuclei: Hepes 10mM, NaCl 50 mM, EDTA 1mM, DTT 1mM, NaPirophosphate 1 mM, NaOrtovanadate 1 mM, Nafluorophosphate 1 mM, PMSF 1 mM, protease inhibitor (Complete, ROCHE). Nuclei are lysed in Tris-Cl pH 7,5 50 mM, NaCl 150 Mm, EDTA 10 mM, DTT 1 mM, protease inhibitors. Nuclear lysate (1 mg) is immunoprecipitated with antibody to HDAC (Upstate), N-Myc, SP1 (Upstate) overnight at 4°C. The day after, specific immunoprecipitated material is incubated with 40µl of slurry-beads protein A, allowing the link between our specific antibody and protein A. The beads with immunocomplexes are washed five times with nuclear lysis buffer + NP40 0,25% and boiled in Laemmli sample buffer for 5 min at 100°C. Eluted proteins are separated by SDS-PAGE and analyzed by Western blot.
For GST pull-down assay HEK293 cells are transfected with pRK7-SP1-HA construct and harvest 48 hrs after transfection. Cell lysates are pre-cleared by incubation with GST-saturated glutathione beads for 1 hr. lysates are incubated with GST-N-Myc 1-88, GST-N-Myc 82-254, GST-N-Myc 249-361 and GST-N-Myc 336-644 for 1 hr at 4°C followed by incubation with glutathione beads for 1hr. bound protein are eluted with sample buffer and subjected to SDS/PAGE and analyzed by Western blot.

SOUTHERN BLOT

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane support. The transfer or a subsequent treatment results in immobilization of the DNA fragments, so the membrane carries a semipermanent reproduction of the banding pattern of the gel. After immobilization, the DNA can be subjected to hybridization analysis, enabling bands with sequence to a labeled probe to be identified. The blotting is performed onto a positive charged nylon membrane with an alkaline buffer. The advantage of this combination is that no post-transfer immobilization step is required, as the positively charged membrane binds DNA irreversibly under alkaline transfer conditions. The method can also be used with neutral nylon membranes but less DNA will be retained.

Digest the DNA samples with appropriate restriction enzymes, run in a agarose gel with appropriate DNA size markers, stain with ethidium bromide, and photograph with a ruler laid alongside the gel so that bend positions can later be identified on the membrane. The gel should contain the minimum agarose concentration needed to resolve bands in the area of interest and should be < 7mm thick. The amount of DNA that must be loaded depends on the relative abundance of the target sequence that will subsequently be sought by hybridization probing. Rinse the gel in distilled water and place in a clean glass dish containing ~10 gel volumes of 0,25 M HCl. Shake slowly on a platform shaker for 30 min at room temperature. This step results in a partial depurination of the DNA fragments, which in turn leads to strand cleavage. The length reduction improves the transfer of longer molecules.pour off the HCl and rinse the gel with distilled water. Add ~10 vol of 0,4M NaOH into the dish and shake slowly on a platform shaker for 20 min. This is the denaturation step. Set up the transfer via downward capillary transfer in a glass dish filled with enough 0,4M

NaOH solution. The transfer pyramid is composed of 2-3 cm of paper towels, Whatman 3MM nylon membrane and gel. An O/N transfer is sufficient for most purposes. Make sure that the reservoir of 0,4M NaOH does not run dry during the transfer. At the end of the transfer remove the paper towels and filter paper and recover the membrane. Rinse the membrane in 2XSSC, place on a sheet of Whatman 3MM filter paper, and allowto air dry. Baking or UV crosslinking is not neede with a positevely charge membrane; in fact UV crosslinking is detrimental. Store the membranes dry between sheets of Whatman 3MM paper for several months at room temperature.

HYBRIDIZATION ANALYSIS OF DNA BLOTTED

Heat pre-hybridization buffer at 65°C and equilibrate the membrane in 50ml of this buffer. Incubate on a rotor for 1hr. Then eliminate this solution and incubate with 10ml of pre-hybridization buffer for 3hrs, adding 1mg of placental DNA previously denatured at 100°C for 5 min. Labell the probe. The Megaprime (Biosciences) systems allow DNA from a variety of sources to be labelled *in vitro* to high specific activity with 32P and other radionuclides. Dissolve the probe to be labelled to a concentration of 2,5-25ng/µl in TE buffer. Place the rquired tubes from the Megaprime system, with the exception of the enzyme, at room temperature to thaw. Leave the enzyme at -15°C to -30°C until required, and return immediately after use. Place 25ng of template DNA into a microcentrifuge tube and to it add 5 µl of primers and the appropriate volume of water to give a total volume of 50µl in the final reaction. Denature by heating to 95-100°C for 5 minutes in a boiling water bath. Spin briefly in a microcentrifuge to bring the contents to the bottom of the tube. Keeping the tube at room temperature and add the nucleotides and reaction buffer followed by radiolabelled dNTPs and enzyme. Mix gently by pipetting up and down and cap the tube. Spin for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube. Incubate at 37°C for 10 minutes and then stop the reaction by the addition of 5µl of 0,2M EDTA. Denature the labelled DNA by heating to 100°C for 5 min, then chill on ice. At the end of pre-hybridization remove the buffer and add 10 ml of hybridization buffer with the denatured probe. Incubate O/N at 65°C and then wash 2-3 times the membrane with 50ml of washing buffer at 65°C for 45 minutes for each washing. Place the membrane in a x-ray film cassette with a sheet of autoradiography film on top of themembrane. Close the cassette and expose at -80°C for 1 week.

TRANSCRIPTIONAL REGULATION OF ABC TRANSPORTERS GENES IN NEUROBLASTOMA CELL LINES.

Some ABC genes, such as *MRP1/ABCC1* and *MRP4/ABCC4* represent predictive prognostic markers of a poor outcome for children neuroblastoma [44, 72]. A strong association between *N-MYC* amplification or overexpression and enhanced levels of *MRP1* and *MRP4* has been demonstrated in neuroblastoma tumours [44, 72]. It has been previously shown that N-Myc regulates the *MRP1* gene, interacting with a putative E-box element in addition to other *cis*-acting factors [75]. It is therefore of interest to investigate whether other members of ABC genes family can be directly regulated by N-Myc oncoprotein. To address this issue we compared transcriptional profile of the 48 human ABC genes with that of *N-MYC*. We used a NB cell line stably transfected with *N-MYC* construct, the transcription of which is under the control of the rTET-inducible expression system (Tet21/N) [296]. The transcriptional level of each ABC gene was evaluated by qRT-PCR during a time course with tetracycline and cluster analysis was performed using CIMminer microarray tool (Fig.1). ABC genes can be grouped in four different clusters, differently correlated with *N-MYC* expression. A first group represents ABC genes, the expression of which don't change during time course with tetracycline; a second group identifies ABC genes, which are positively correlated with *N-MYC* down-regulation; whereas a third group of ABC genes presents a transcriptional profile contro-correlated with *N-MYC* expression. Finally, transcriptional profile of several ABC transporters genes are not here represented because they are not expressed in Tet21/N cell line. Particularly interesting is that three ABC transporters, and precisely *MRP1/ABCC1*, *MRP4/ABCC4* and *MRP3/ABCC3*, have great clinical relevance. High levels of *MRP1/ABCC1* and *MRP4/ABCC4*, associated with low levels of *MRP3/ABCC3* represent the most predictive marker of poor clinical outcome in neuroblastoma (Haber et al, data unpublished).

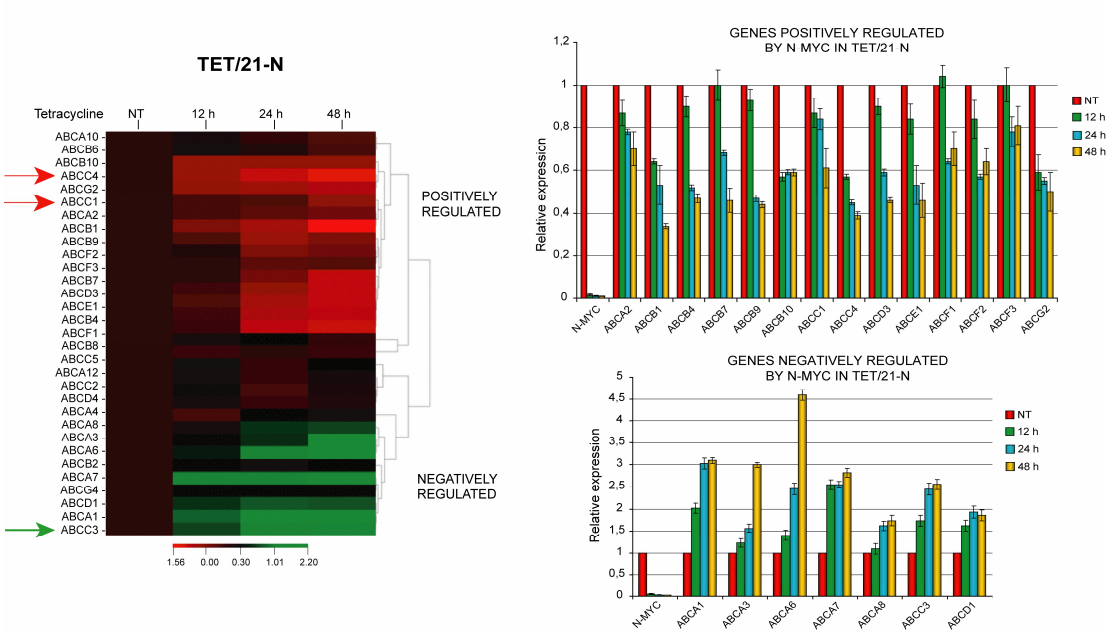


Figure 1. Relative mRNA expression of ABC transporter genes was determined in TET/21-N neuroblastoma cells as a function of N-Myc downregulation. (A) Cluster analysis performed using the CIMMiner microarray tool. (B) Relative expression of ABC transporter genes positively (top) or

negatively (bottom) regulated by N-MYC in TET/21-N cells at different time points of tetracycline treatment.

Although MRP3 shows structure homology with MRP1 and MRP4 transporters, normally involved in chemoresistance, there is no evidence of a clear association between MRP3 and drug resistance phenomenon. To verify that ABC transcription profile was also correlated with that of *N-MYC* in other cell lines, their mRNA levels were evaluated in five human neuroblastoma cell lines, which express different levels of N-Myc. Figure 2b shows a cluster analysis diagram of ABC expression across these five neuroblastoma cell lines. ABC genes upregulated by N-Myc were highly expressed in Lan-1 and SK-N-BE where the oncogene is amplified or overexpressed, whereas their expression was lower in SH-SY-5Y, SK-N-SH and SHEP (with low level of *N-MYC*). Otherwise, *MRP3/ABCC3* was expressed in those cells, where *N-MYC* expression is low, while its transcription resulted decreased when *N-MYC* was amplified or overexpressed.

Based on the results of the qRT-PCR analysis, we therefore propose that N-Myc is a transcriptional activator for several ABC genes and a transcriptional repressor for *MRP3/ABCC3*.

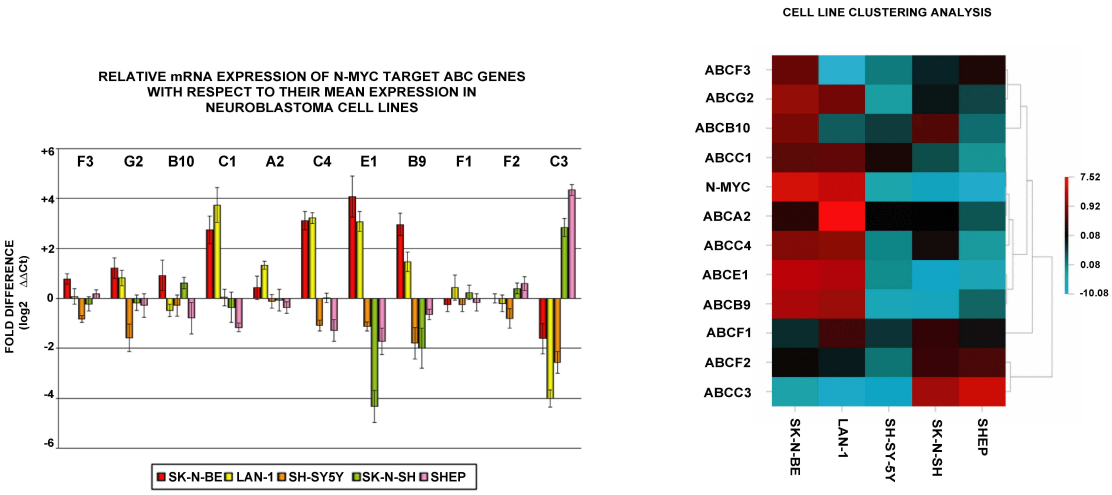


Figure 2. mRNA expression of ABC transporters was determined in five distinct human neuroblastoma cell lines and correlated with that of N-MYC. Results show that in most cases transporter gene expression was significantly higher in cells overexpressing N-MYC.

N-Myc AFFECTS DYE AND DRUG EFFLUX IN NEUROBLASTOMA CELL LINES

To determine how N-Myc can influence drug response we have compared the sensitivity of several neuroblastoma cell lines to chemoterapeutics commonly used in therapy. Cells were incubated with increasing concentration of some chemotherapeutic agents, such as vincristine and doxorubicin, which are transported by MDR1, MRP1 and BCRP transporters. Furthermore, doxorubicin and vincristine forms an integral part of the therapy of many childhood solid tumours, including the therapy of neuroblastoma. High-risk neuroblastoma tumours, highly correlated with *N-MYC* amplification, demonstrated drug resistance against these compounds. Therefore, it was important to understand whether N-Myc might induce cellular drug resistance against these agents.

Cell survival to these compounds was measured by MTT assay. Following 72h of continuous exposure to each compound, the cell lines with amplification or overexpression of *N-MYC* displayed an ID50 of more than five times higher than the neuroblastoma cell lines with low expression of *N-MYC* itself (Fig. 3).

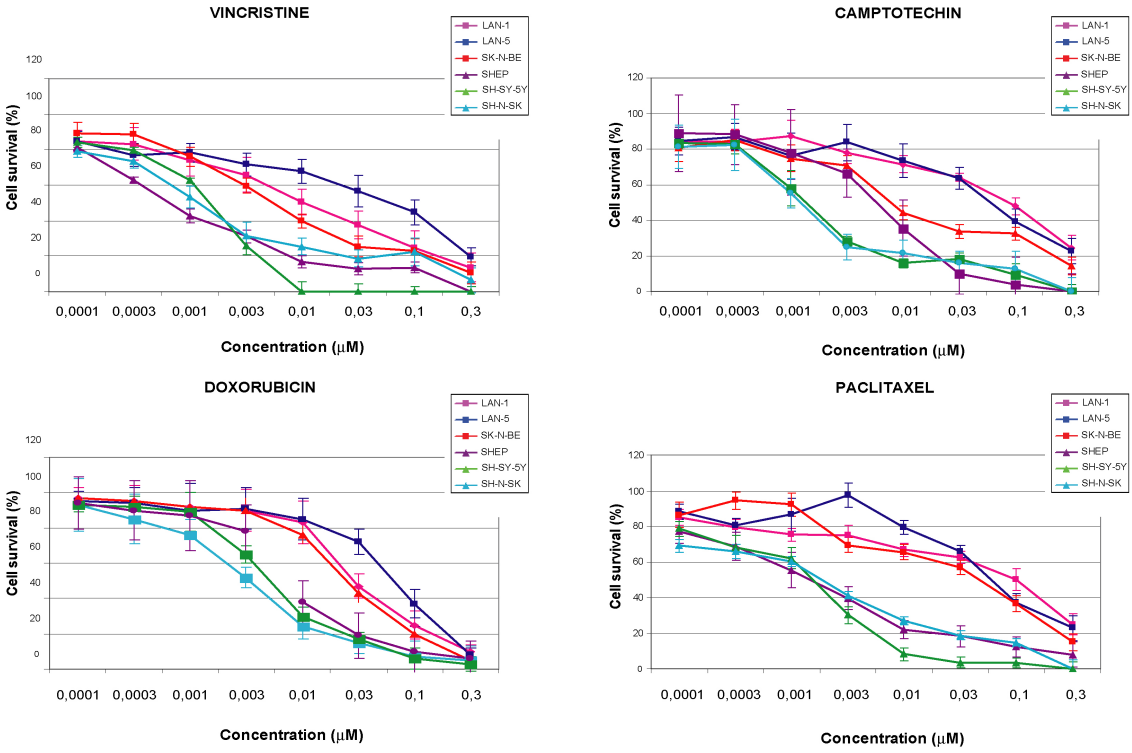


Figure 3. *N-MYC* expression levels correlate with cell resistance to drugs.

In order to understand whether the increased resistance in NB cell lines with *N-MYC* overexpression could be associated with increased efflux activity, we evaluated the accumulation of two dyes, Rhodamine and DiOC2, in Tet21/N in absence of tetracycline (Tet21/N -) or following its addition (Tet21/N+).
DiOC2 is highly specific for Mdr1, and is not transported by the related multidrug resistance protein, MRP1 [297]. Rhodamine 123 is effluxed by MDR1 and to a lesser extent by MRP1, and thus serves as a more broad indicator of total cellular efflux activity. Another member of the ABC family, breast cancer resistance protein (BCRP), weakly transports DiOC2, but does not transport Rhodamine 123 [298]. We evaluated the functional activity of the MDR1, MRP1 and BCRP membrane pumps by directly measuring the relative fluorescence of cell populations that actively extrude fluorescent multidrug resistance transport substrates. The efflux activity of MDR1 and its relatives is highly temperature sensitive. They optimally function near 37ºC, but are effectively inactive at 4ºC. ABC transporters-expressing cells preloaded with MDR1 fluorescent substrates retain the dye and consequently have high fluorescence when incubated at 4ºC. Conversely, cells incubated at 37ºC more readily efflux the dye and show reduced fluorescence (37ºC + DMSO,). A specific inhibitor, vinblastine, was also used as a substrate for MDR1 and it competitively blocks efflux of DiOC2(3) and Rhodamine 123. Inclusion of excess vinblastine in the efflux reaction at 37ºC therefore results in high fluorescence (37ºC + vinblastine).

Consistent with the results of cytotoxicity assay, N-Myc can influence the efflux of specific ABC transporters substrates, directly influencing their transcriptional activity. Indeed, a highly significant reduction in the amount of each specific dye retained in the cell was observed in cells with high *N-MYC* expression but not in cells where *N-MYC* is silenced (Fig. 4). In presence of vinblastine, the fluorescence of cell populations resulted unchanged, demonstrating that the efflux of these dyes is specifically controlled by MDR1, MRP1 and BCRP.

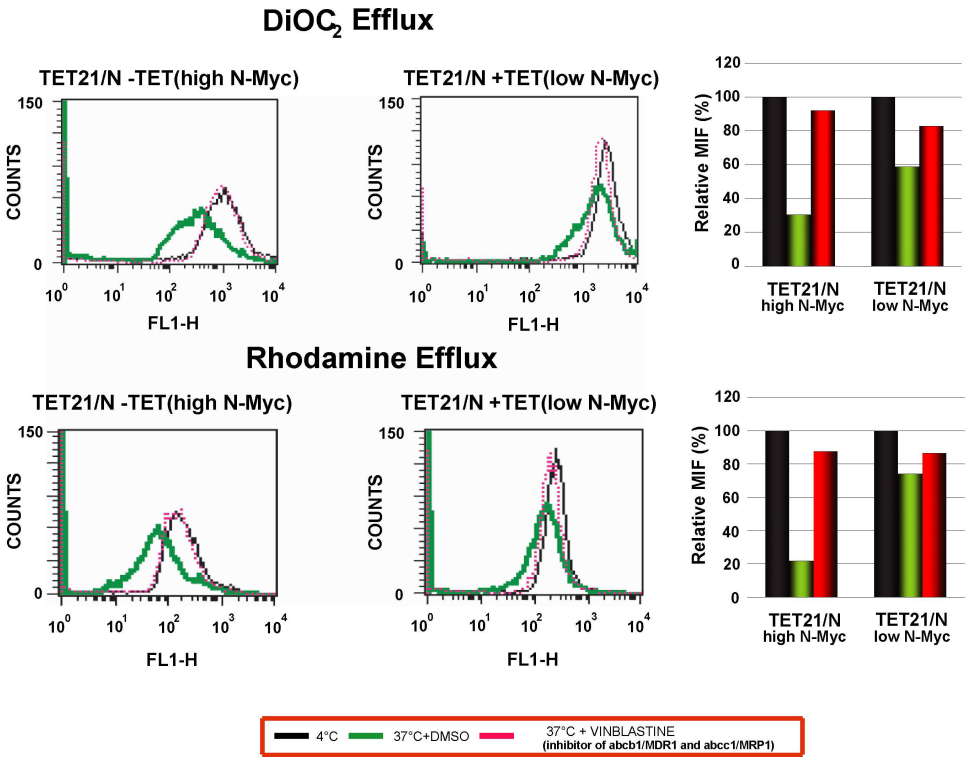


Figure 4. The efflux activity of ABCB1/MDR1, ABCC1/MRP1 and ABCG2/BCRP was compared between TET/21-N –TET (high N-Myc) and TET/21N +TET (low N-Myc).

N-Myc REGULATES TRANSCRIPTION OF ABC GENS BY BINDING DIRECTLY TO THEIR PROMOTERS

To confirm the direct association of N-MYC with the promoters of ABC genes up-regulated by N-Myc itself, ChIP studies were performed in Tet21/N- cell line. Each ABC promoter was evaluated for the presence of canonical and non-canonical Myc binding sites (E-Box) by bioinformatic tools. We therefore searched for E-Box motifs located within a distance of 2 Kb on either side of the transcriptional start site. Immunoprecipitated DNA was analyzed by qPCR using pairs of primer flanking the specific binding sites. ChIP results indicated that heterodimer N-Myc/Max binds the promoters of tested ABC genes in close proximity to the transcriptional start site.

abcb1 represents an exception, because its promoters was not bound by N-Myc, thus it could be proposed as a secondary target of N-Myc oncoprotein (Fig. 5).
ChIP analysis confirmed that N-Myc is directly associated with nine ABC genes (*ABCA2*, *ABCB9*, *ABCB10*, *ABCC1*, *ABCC4*, *ABCE1*, *ABCF1*, *ABCF2* and *ABCG2*). However, the binding of Myc at its consensus sequence appears to be required, but not always sufficient for transcriptional activation (Frank et al.; Fernandez et al.).

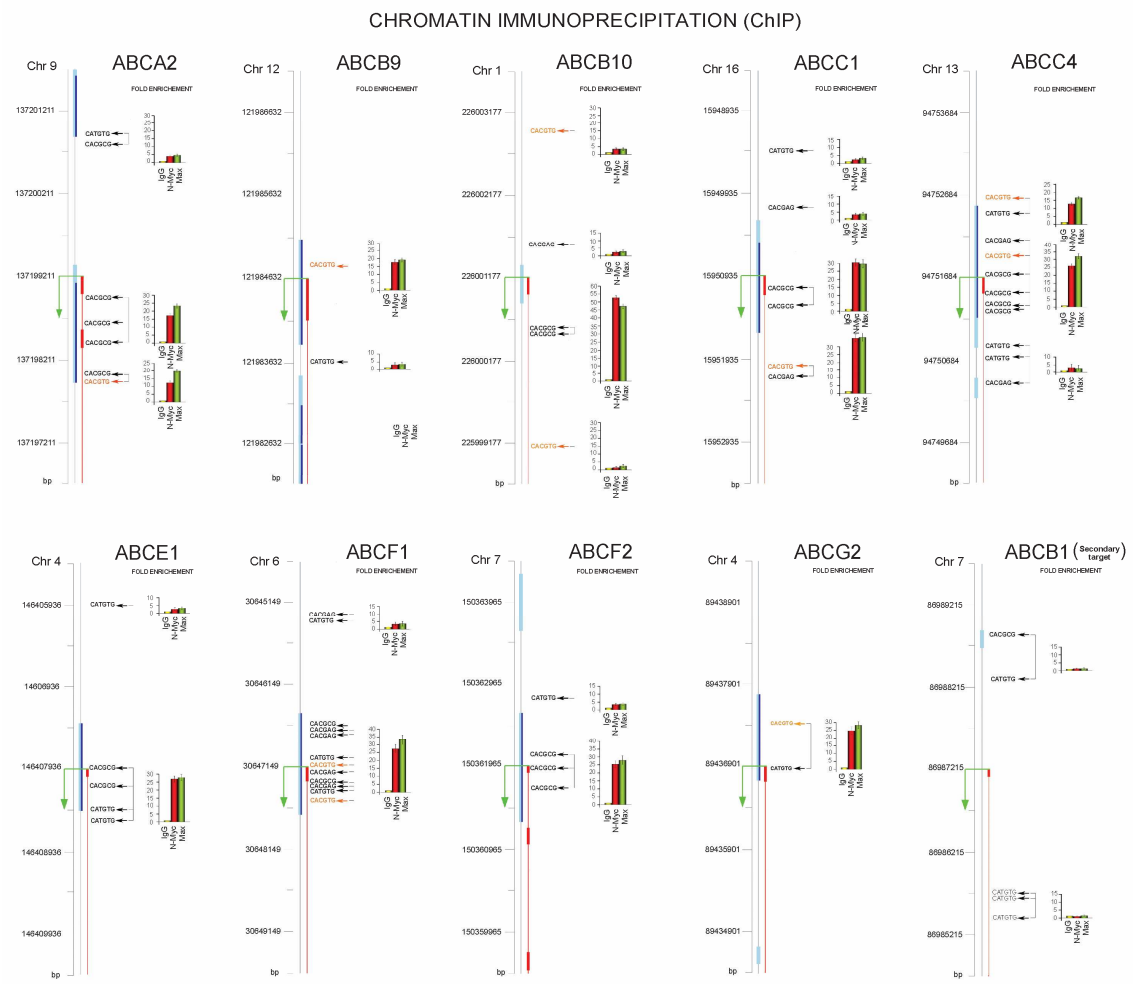


Figure 5. N-Myc is a direct regulator of several ABC transporter genes and binds ABC transporter promoters in close proximity to the transcription start site.

To demonstrate that N-Myc binding to ABC promoters directly affected their transcriptional activity, we performed a luciferase assay. For each ABC gene, we have cloned the promoter into luciferase expression vector. Luciferase assay was performed in Tet21/N, and we have evaluated promoter activity in dependence of N-Myc expression. For ABC genes directly bound by N-Myc, luciferase assay displayed a decreased promoter activity when *N-MYC* was downregulated, by suggesting that the binding of the oncoprotein is necessary for their transcriptional activation (Fig. 6).

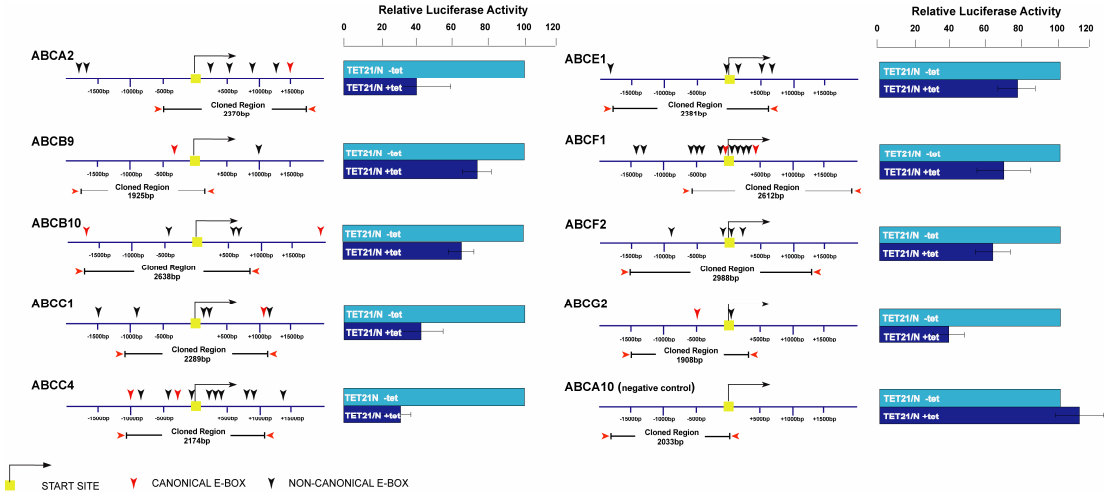


Figure 6. c-Myc role on ABC gene transcription is recapitulated by transient transfection assay. ABC transporter promoters were cloned into a luciferase reporter vector. Constructs were testet in Tet21/N cells as function of *c-MYC* expression (+/- Tetracycline). The *ABCA10* gene was used as a negative control in that it does not respond to N-Myc.

N-Myc REPRESSES THE *ABCC3* PROMOTER BY INTERACTION WITH TRANSCRIPTION FACTOR SP1

Myc exerts its biological functions at least in part through the transcriptional regulation of target genes. The best understood function of the protein is its ability to activate genes through recruitment of several activating cofactors to DNA [299]. Interestingly, MYC can acts also as transcriptional repressor. Indeed, detailed evidence has accumulated to suggest that cell-cycle inhibitory genes and other genes such as *MAD4* and *NDRG2* are repressed by c-Myc [299]. Three alternate mechanisms have been proposed that might explain repression by c-Myc. The Myc mediated repression does not require DNA binding, but interaction with some transcriptional activators such as Miz-1, Sp-1 and SMAD. These factors, interacting with Myc, lose their transactivator function by determining an inhibition of gene transcription [300]. Based on *ABCC3* transcriptional profile in Tet21/N and in some neuroblastoma cell lines (Fig.1 and 2) we proposed that N-Myc might regulate negatively its expression acting as a repressor. Since there is a significant functional overlapping between c- and N-Myc, we hypothesized that also N-Myc may repress transcription through a mechanism similar to that of c-Myc.

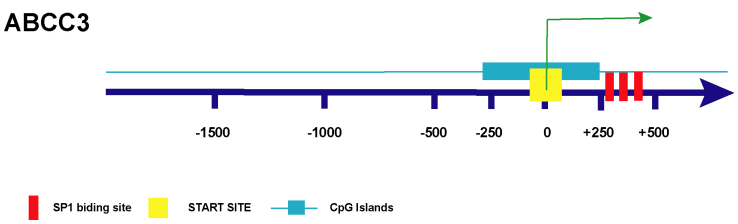


Figure 7. Schematic representation of *ABCC3* gene promoter. The localization of the CpG island is indicated by sky-blue line, while Sp1 binding sites are represented by the vertical red lines.

Thus, we have analyzed *ABCC3* promoters for the presence of Sp-1 binding sites. *ABCC3* promoter doesn't contain E-Boxes in close proximity to its transcriptional start site, whereas GC boxes and Inr element are located around its start site (Fig. 7).
Thus, we have evaluated the binding of Sp1 to its cognate sites and we have checked if also N-Myc could bind *ABCC3* promoter on GC boxes through its specific interaction with Sp1. We have tested this hypothesis in a Dual-Step ChIP assay. Conventional ChIP technique, using a single formaldehyde cross-linking step, is insufficient to detect the binding of transcription factors that participate to the regulation of gene transcription by interacting with other proteins, associated with promoters, but not with DNA. To overcome this problem, we thought of using the cross-linking agent DSG (Di(N-succinimidyl)glutarate) in addition to formaldehyde that may improve the formation of covalent links between proteins and stabilize the association of proteins to DNA, though not directly bound to it. Based on other studies we set up a dual cross-linking ChIP protocol that we have successfully employed to improve immuno-precipitation of complexes in which tested factors are not in direct contact with DNA. Specific *ABCC3* promoter region supposed to be engaged with Sp1 and N-MYC were analyzed by qPCR. We found that either Sp1 or N-Myc bind *ABCC3* promoter on a specific region located between +200 and +500bp and containing three GC boxes (Fig. 8). This finding, together with the evidence that we was not able to detect N-Myc binding on *ABCC3* promoter using conventional ChIP (data not shown), supported the hypothesis that N-Myc can repress *ABCC3* transcription through a direct interaction with Sp1 but not with its DNA promoter.

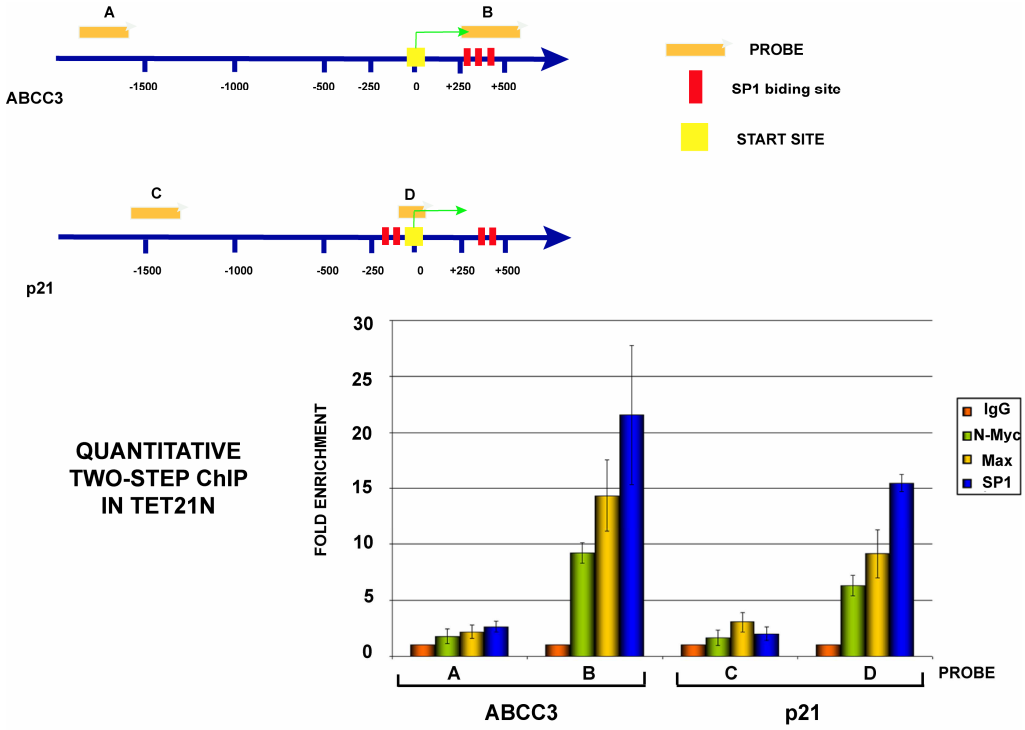


Figure 8. N-Myc represses *ABCC3* gene transcription. Dual cross-linking ChIP and quantitative PCR were applied to Tet21/N- cells. Fold enrichment of a given DNA region immunoprecipitated with anti N-Myc, Max or Sp1 antibodies was calculated as the ratio between the enrichment obtained with a specific antibody and that obtained with the pre-immune serum. p21 represents positive control for N-Myc mediated repression.

To investigate the role that these GC box elements play in the regulation of *ABCC3*, reporter constructs were generated and transiently transfected into Tet21/N cells. The highest level of *ABCC3* promoter activity was observed with the full length promoter reporter construct, which contains all three GC boxes, in Tet21/N+ cells, where *N-MYC* is downregulated. On contrary, *ABCC3* promoter activity resulted decreased when *N-MYC* was switched on (Tet21/N-), indicating that N-Myc represses *ABCC3* promoter. When transfection assay was performed with promoter reporter construct that doesn't contain these three GC boxes, the promoter activity was lower and didn't change in dependence of *N-MYC* expression. The effects of increased *N-MYC* expression on *ABCC3* promoter activity were examined also in a co-transfection assay with a plasmid which contained *SP1* coding sequence under the control of a CMV promoter (pRK7-*SP1*). Also in this case *N-Myc* was able to repress the wild type promoter activity, but not influenced the activity of deleted construct, indicating that the region of *ABCC3* promoter which contains the three GC boxes, located between +250 and +500bp, is required for N-Myc mediated repression (Fig. 9).

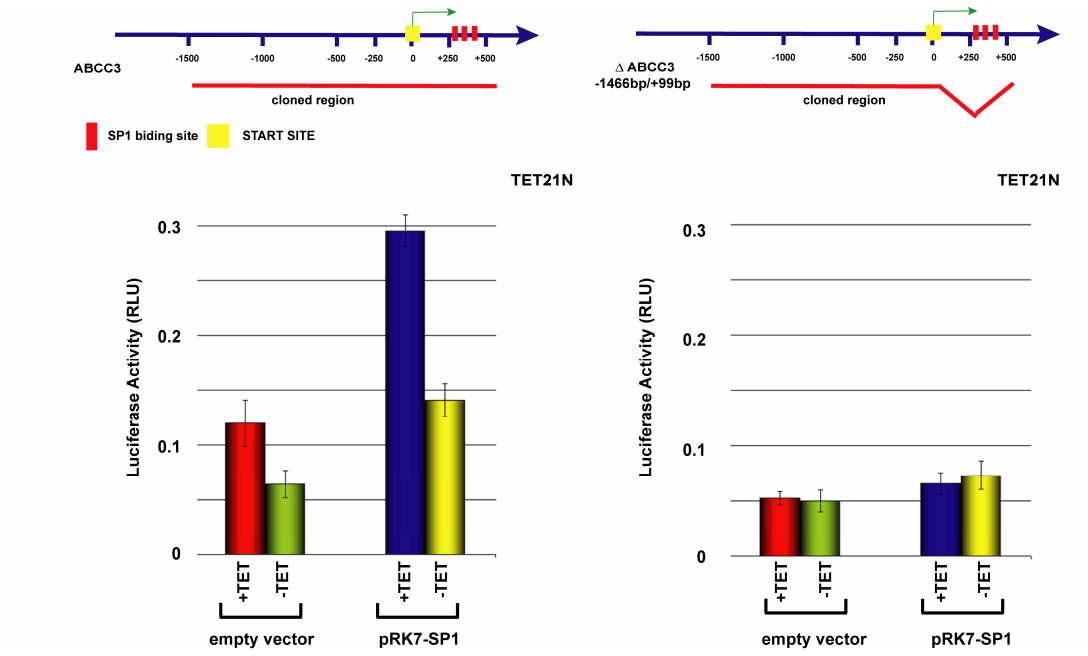


Figure 9. *ABCC3* promoter requires the Sp1 core region for N-myc repression. Luciferase activity of the two reporters ABCC3 and ΔABCC3 was determined in presence (- TET) or absence (+TET) of N-Myc expression and normalized to that of renilla

To determine whether N-Myc repression of the *ABCC3* promoter is based on interactions between N-Myc and the transcription factor Sp-1, we co-transfected N-Myc-FLAG vector and HA-Sp1 vector in HEK-293 cell line and performed immunoprecipitations using antibody against HA-Sp1 followed by immunoblotting with antibodies against N-Myc-FLAG. We also performed co-immunoprecipitation assay in SK-N-BE in order to detect endogenous interaction. Nuclear extracts were prepared and subjected to immunoprecipitation with Sp1 antibody, followed by

immunoblotting with N-Myc antibody. Using these assays we have observed association between N-Myc and Sp1 (Fig. 10a).
To identify the region of N-Myc that is responsible for interaction with Sp1, we used GST-fusion protein containing four different domains of N-Myc (Δ1-88, Δ82-254, Δ249-361 and Δ336-644). These GST-fusion proteins were used in pull-down experiments with extract from HEK-293 cells transfected with the plasmid expressing SP1 (pRK7-SP1). We identified that two N-Myc domains (Δ82-254 and Δ336-644) are involved in interaction with SP1 (Fig. 10b)

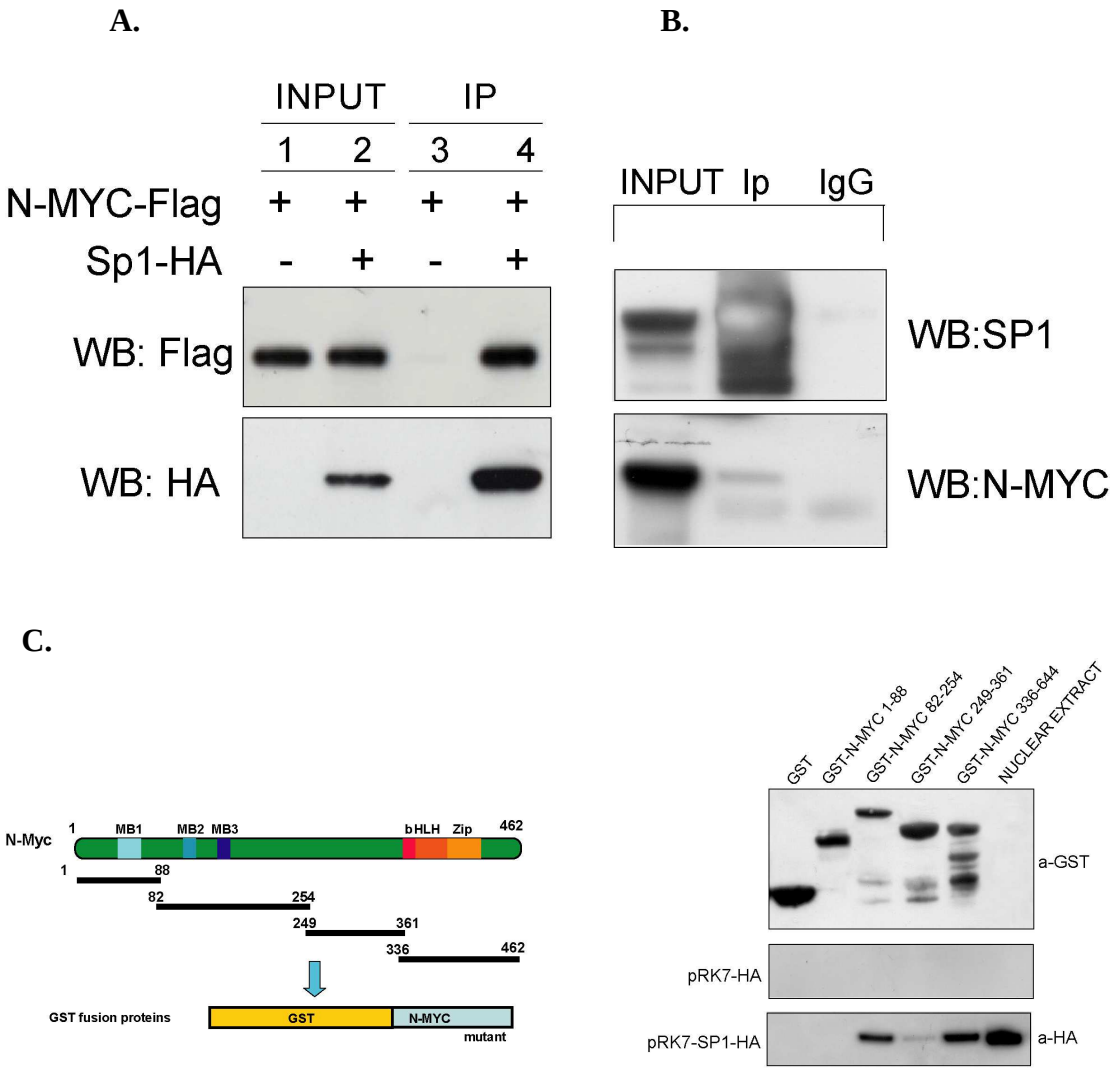


Figure 10. N-Myc is associated with Sp1. **a)** Immunoprecipitation (IP) with HA antibody was performed with nuclear extracts from HEK-293 cells transiently tranfected with mammalian expression constructs encoding N-Myc-FLAG and Sp1-HA, followed by immunoblotting with monoclonal anti-M2 Flag antibody **b).** Immunoprecipitation (IP) with Sp1 antibody was performed with nuclear extracts from SK-N-BE neuroblastoma cell line, followed by immunoblotting with monoclonal anti-N-Myc antibody. **c).** HEK293 cells were transfected with pRK7-Sp1-HA or pRK7-HA and harvested 48h after transfection. Pull-down assays were performed by incubating lysates with GST or GST-N-MycΔ1-88, GST-N-MycΔ82-254, GST-N-MycΔ249-361, GST-N-MycΔ336-644 for 1h at 4°C followed by incubation with glutathione beads for 1h. bound proteins were subjected to SDS/PAGE followed by immuniblotting with anti-HA antibody.

c-Myc IS ALSO A POSITIVE AND DIRECT REGULATOR OF SEVERAL ABC TRANSPORTER GENES.

Many haematological malignancies are often associated also with alterations in the level of *c-MYC* expression. Understanding how c-Myc can promote the leukemic phenotype will provide novel tools for designing more efficient drugs to promote regression of leukemic tumours. We have tested the hypothesis that, like N-Myc, c-Myc could play a critical role in the insurgence of drug resistance phenomenon in CML and AML, may be controlling the expression of ABC drug transporter genes.
To achieve this aim, ABC genes expression was monitored in HL60, a human promyelocytic cell line in which *c-MYC* is overexpressed and can be turned off by treating cells with DMSO, and in P493, a lymphoblastoid cell line modified to overexpress *c-MYC* under a tet-off promoter. In these cell lines, we have examined the expression level of all forty-eight human ABC drug transporters as a function of *c-MYC* silencing. Their transcription profiles was determined by qRT-PCR and described by cluster analysis (Fig. 11). Our results demonstrated that c-Myc affected the transcription of several ABC genes, such as *ABCA2*, *ABCB10*, *ABCC1*, *ABCC4*, *ABCE1*, *ABCF1*, *ABCF2* and *ABCF3*, a majority of which, has been found implicated in chemoresistance.

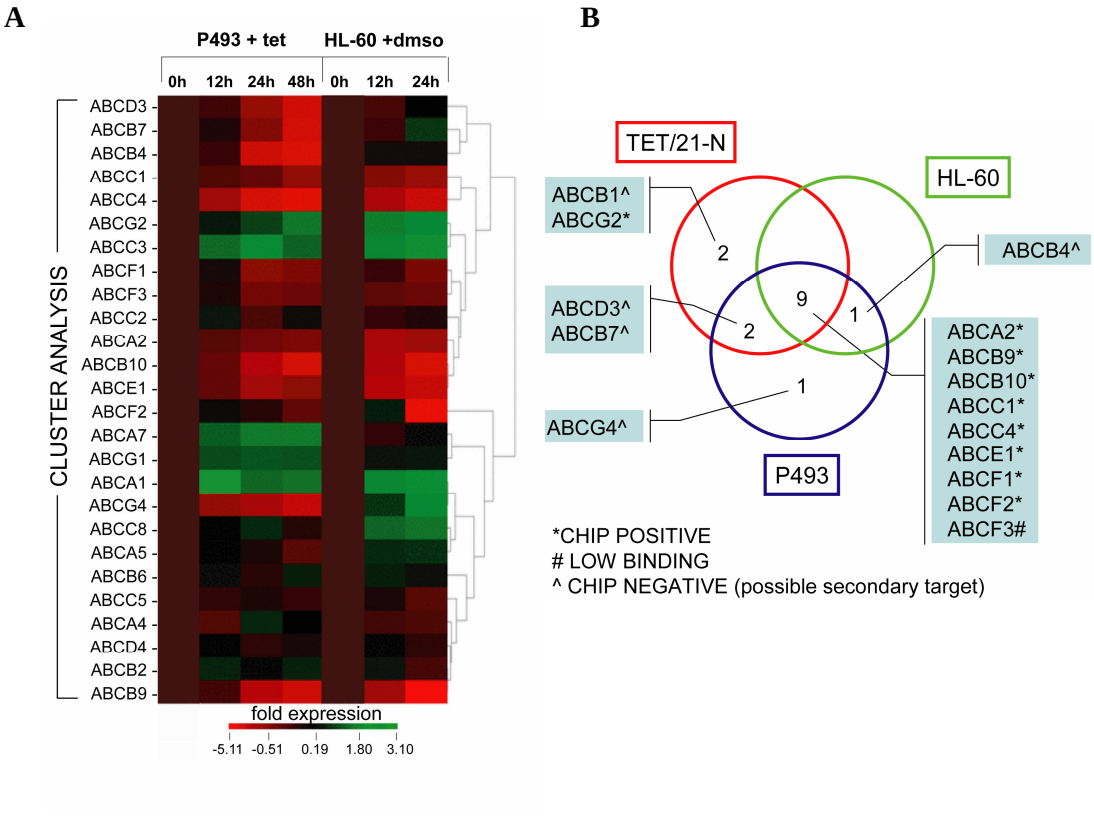


Figure 11. Transcriptional profiles of ABC drug transporters genea as function of *c-MYC* expression. The analysis was performed in P493 and HL-60 cell lines. Relative mRNA expression of all 48 human ABC transporter genes was determined by qRT-PCR as a function of *c-MYC* silencing. The analysis describes those genes that are positively or negatively regulated by c-Myc or for which *c-MYC* silencing has no effect on their expression. Genes, the expression of which is totally absent in the two cell lines, are noy listed in the cluster study.

Furthermore, performing ChIP assay, we have demonstrated a direct binding of c-Myc to the promoters of the ABC genes positively regulated by c-Myc itself (Fig. 12).

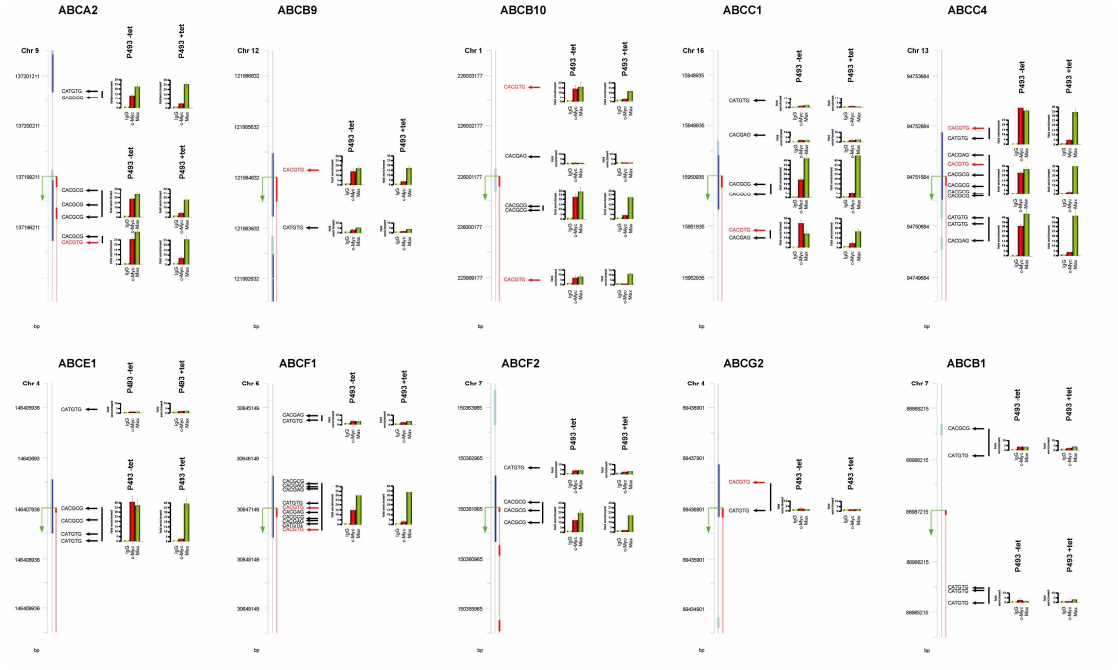


Figure 12. c-Myc is a direct transcriptional regulator of ABC transporter genes.

DNA METHYLATION AND CHROMATIN STRUCURE MAY PLAY A KEY ROLE IN MULTIDRUG RESISTANCE IN CANCER.

These data were compared to those obtained in Tet21/N cell line and the results show that both c-Myc and N-Myc appear to control a large number of ABC transporter genes. Venn diagram (Fig. 11b) shows that nine common genes are positively regulated by c-Myc and N-Myc. Although *ABCB1* and *ABCG2* seemed not to be regulated by c-Myc, however, these genes were not expressed either in P493 or in HL60 and for this reason we couldn't exclude that c-Myc was involved in their transcriptional control. Epigenetic events may influence *ABCB1* and *ABCG2* transcriptional regulation. These two genes are expressed at high levels in haematopoietic stem cells, but they are turned off in most committed progenitor and mature blood cells [91]. Several studies have demonstrated that methylation of the *ABCB1* promoter was linked with a lack of *ABCB1* expression in cell lines and samples from patients that were diagnosed with AML, acute lymphocytic leukaemia, chronic lymphocytic leukaemia, colorectal cancer and bladder cancer [200]. Thus, *ABCB1* promoter methylation imposes a restrictive chromatin environment. However, when treated with a DNA demethylating agent (5'-Aza-2'-deoxycytine), P493 cells showed reactivation of several ABC genes, including *ABCB1* and *ABCG2*, otherwise silent (Fig. 13).

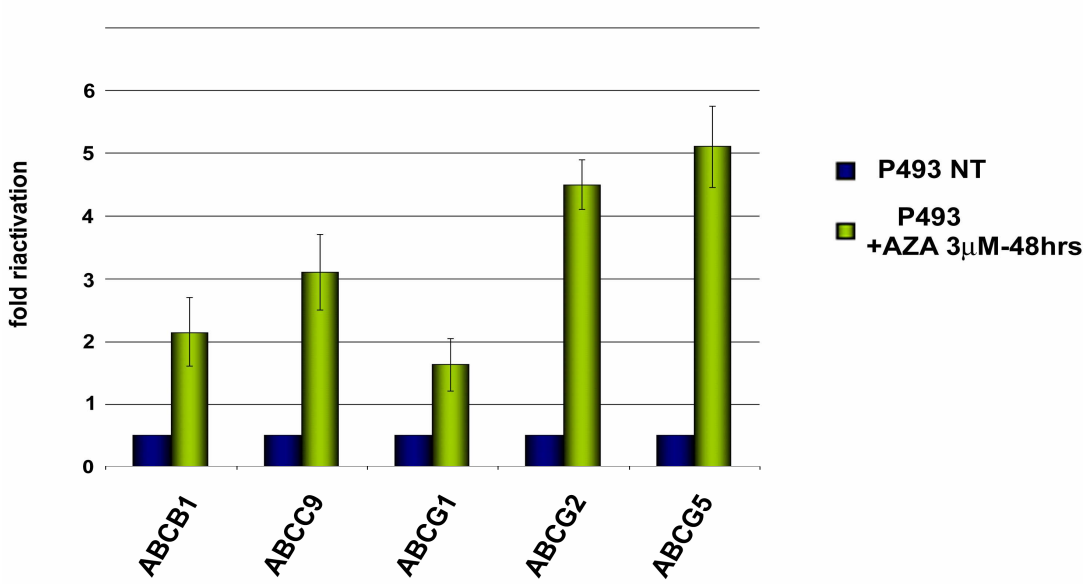


Figure 13. Transcriptionale reactivation of some ABC genes following the treatment with 3μM of 5'-Aza-2'-deoxycytine for 72 h in P493 cell line.

Based on the evidence that Abcg2 plays a key role in regulating drug resistance in leukaemia malignancies, we focused our attention on the chromatin structure of its promoter. The reactivation of *ABCG2*, following treatment with 5'-Aza-2'-deoxycytine, allows us to hypothesize that its promoter and more specifically the Myc binding sites present in it, are methylated in P493 cells, thus inhibiting Myc binding as previously proposed. In order to evaluate the methylation of *ABCG2* promoter in P493 and HL60 cell lines we have performed a Southern Blot analysis. We have digested genomic DNA with MspI, which recognizes and cleaves CCGG elements independently of their methylation status; HpaII, which cleaves the same sequence only when demethylated; and PmlI which recognizes Myc binding site (CACGTG) but is sensitive to methylation status of this sequence. The results showed that, in these cell lines, *ABCG2* promoter was hypermethylated, even if Myc binding site remained unmethylated (Fig. 14).

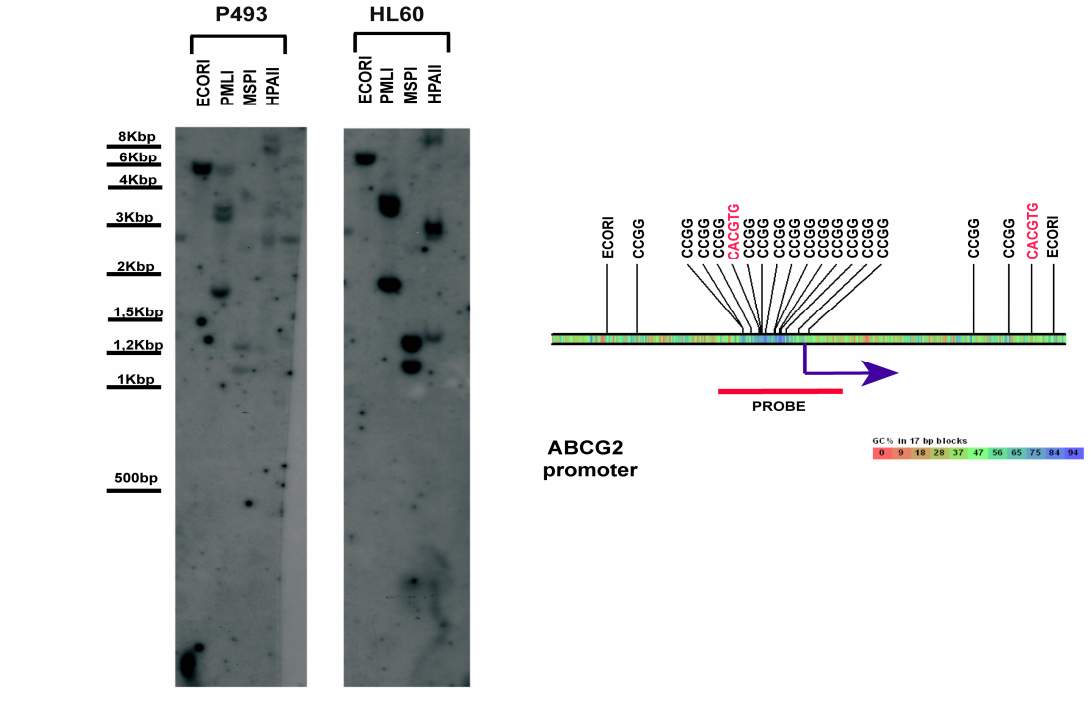


Figure 14. Southern Blot analysis of *ABCG2* promoter. Genomic DNA (15 µg) digested with the indicated enzymes was electrophoresed on an agarose gel and analyzed by Southern blot hybridization. The *ABCG2* probe extends from the promoter region to within the coding sequence. Position of the *Hpa*II/*Msp*I sites are shown schematically over the *ABCG2* gene diagram. The digestion profile of genomic DNA with MspI was quite different from that of HpaII, indicating that several CpG sites were methylated. We digested P493 and HL60 genomic DNA also with PmlI, which cleaves E-Box site (CACGTG) only when demethylated. The digestion profile for PmlI, if compared with EcoRI digestion profile, showed that the only one N-Myc binding site was unmethylated.

c-Myc CONTROLS EXPRESSION OF ABC DRUG TRANSPORTERS IN CD34+ HEMATOPOIETIC PROGENITORS

The treatment of haematological malignancies appears to be unsatisfactory in child and adult patients with acute myeloid leukaemia. An important problem in the treatment of acute myeloid leukaemia is caused by a pre-existent or acquired drug resistance. P-gp/MDR1 is strictly associated with unfavourable outcome in paediatric ALL patients, whereas MRP1 and BCRP do not seem to play a major role in this type of leukaemia. Also in the treatment of chronic myeloid leukaemia several patients does not respond and others lose response during treatment. CML is linked to a acquired genetic abnormality. Identification of the BCR-ABL kinase fusion protein as a central player in the pathogenesis of CML leaded to develop of a selective inhibitor of this fusion protein, named imatinib. The resistance to this chemotherapeutic agent can be due to overexpression/amplification of BCR-ABL kinase or to emergence of its mutant isoforms [301]. In addition, BCR-ABL itself may regulate *ABCG2* expression via AKT activity, suggesting that ABCG2 may be a mediator of imatinib resistance [302]. Moreover also the overexpression of *ABCB1* and its protein seems to be responsible for resistance to imatinib and inhibitors of this transporter are able to restore the drug sensitivity [303]. Thus, like AML, also CML presents a drug resistance strictly related to ABC transporters expression. Furthermore, we have investigated the expression of *c-MYC* and ABC genes in chronic myeloid leukaemia (CML) in order to verify whether high expression levels of *c-MYC* may affect transcription of ABC

drug transporters also in CML cells. CML is characterized by the Philadelphia (Ph) chromosome translocation, which generates *BCR-ABL* fusion gene. This rearrangements is detected in about 95% of patients with CML and, because of its constitutively activated tyrosine kinase activity, it is considered to be the causative molecular abnormality in CML. BCR-ABL may positively regulate c-Myc expression [304]. CML is characterized by high levels of *c-MYC* expression and a large expansion of the CD34+ stem cell population in the peripheral blood [305, 306]. We have performed a transcriptional analysis of *c-MYC* and ABC genes (qRT-PCR) in CD34+ cells from newly diagnosed chronic phase (CP)-CML patients, and we have found that several ABC genes, particularly *ABCC1* and *ABCC4*, were overexpressed in CD34+ population if compared to entire population of mononuclear cells from which stem cells have been purified (Fig. 15).

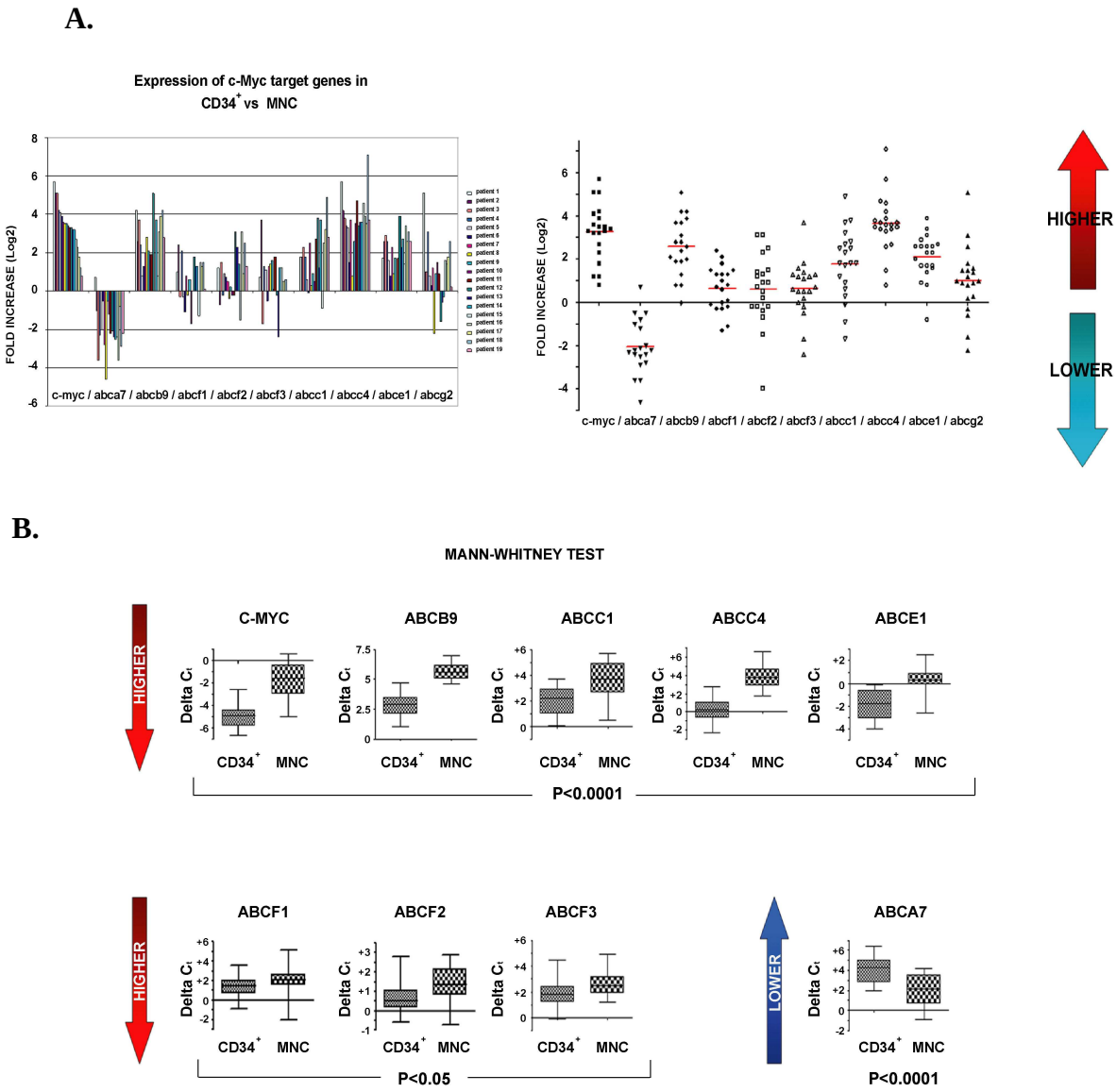


Figure 15. c-Myc controls expression of ABC drug transporters in CD34+ hematopoietic progenitors. **a)** Relative expression of c-MYC and ABC genes of CD34+ cell population was compared to that of the entire population of mononucleated cells (MNC). Each colored bar (up) or dots (below) corresponds to a single patient. ABCA7 which has been used as a negative control results to be repressed in CD34+ cells as compared to MNC cells. **b)** Mann-Whitney statistical test applied to results of panel A confirms that the expression level of analysed genes is significantly different in the

two cell populations . Results are reported as ΔCt values which were calculated as the difference between Ct of the ABC mRNA and Ct of the GUSB mRNA used for normalization of qRT-PCR.

Particularly interesting is that *ABCG2* is expressed in CD34+ population and its expression is correlated to that of *c-MYC*. This result indicated that *ABCG2* promoter is probably demethylated in CD34+ cell population of CML patients, and that in this condition c-Myc is able to act directly on its transcription. Finally, through ChIP assay, we have confirmed that c-Myc was physically associated with promoters of ABC drug transporter genes in CML or AML derived cells (Fig. 16).

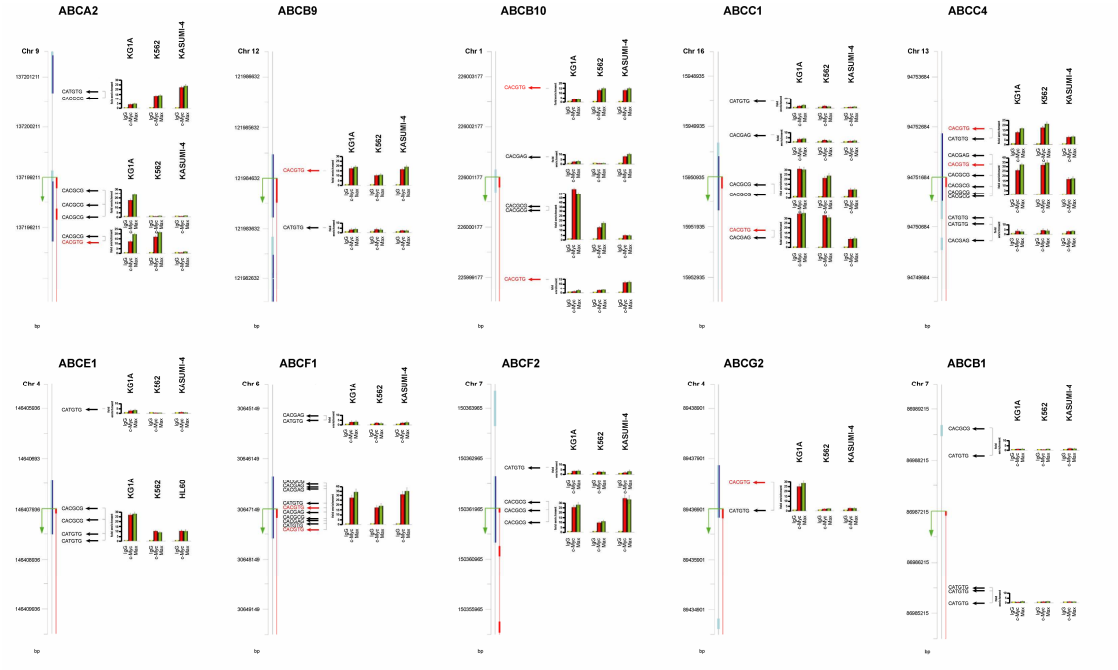


Figure 16. c-Myc is physically associated with promoters of ABC drug transporter genes in CML derived cell lines. To demonstrate that c-Myc can directly control transcription of ABC tranporter genes in CML we have performed ChIP in three distinct cell lines: KG1a derived from AML and expressing the CD34 antigen (control); K562 Ph+, but not expressing the CD34 antigen; Kasumi-4 Ph+ and expressing the CD34 antigen.

It is remarkable that *ABCG2* is expressed in KG1a cell line, derived from AML and expressing the CD34+ antigen. These cells are blocked at an early stage of development and remain in the proliferative pool, saving parameters of staminality. For this reason, *ABCG2* promoter remained unmethylated, allowing the c-Myc binding to its cognate site.

Resistance to chemotherapeutic agents is a major obstacle for successful treatment of cancer. The failure of the curative treatment of cancer patients often occurs as a result of intrinsic or acquired drug resistance of the tumours to chemotherapeutic agents. The resistance of tumours occurs not only to a single cytotoxic drug used, but also occurs as a cross-resistance to a whole range of drugs with different structures and cellular targets. This phenomenon is called multiple drug resistance (MDR). Multidrug resistance (MDR) severely limits the effectiveness of chemotherapy in a variety of common malignancies and is responsible for the overall poor efficacy of cancer chemotherapy [116].

Therefore, understanding how chemoresistance develops and eventually how it can be contrasted becomes crucial to fight cancer effectively. Chemoresistance of cancer cells is in part caused by misregulation of the activity of membrane proteins, named ATP-binding cassette transporters, responsible for the efflux of chemotherapeutic agents in cancer cells [9, 34]. Such misregulation may be achieved by mutations affecting the biophysical and biochemical properties of the transporters or by an increase in their expression level. The human genome codes for forty-eight functional ABC transporter genes, which can be grouped into seven subsets (from A to G) based on their degree of sequence homology [307]. A common strategy adopted to overcome drug resistance in tumours is based on the administration of compounds that inhibit ABC transporters in association with chemotherapeutic agents, normally used in therapy [117]. Although these ABC inhibitors are not toxic themselves, they might inhibit ABC transporters also in normal tissues, enhancing adverse effects of anticancer drugs. Side effects due to modulation of ABC transporters in normal tissues, especially blood-brain barrier should be monitored carefully to avoid neurological response. Thus, considering the limited success of available treatment modalities for the therapy of multidrug-resistant tumour cells, alternative and complementary strategies need to be developed. Antisense oligonucleotide technologies and oncolytic adenovirus strategy represent two novel approaches to struggle with MDR mechanisms and are based on the capability to restore normal transcriptional levels of ABC transporters [193]. For this reason, establishing how the forty-eight human ABC genes are regulated at transcription level and which transcription factors concur to such a control, is crucial to understanding the role of ABC transporters in physiological as well as in pathological contexts, such as cancer. Although many studies have focused on the transcriptional regulation of the *ABCB1/MDR1* gene, which encodes the P-glycoprotein [76], yet very little is known about the molecular mechanisms underlying transcription of the large family of ABC transporter genes. A recent study by Gottesman and colleagues has proposed that the definition of the transcription profile of all forty-eight genes may be important in predicting the possible arising of a multidrug resistance phenotype in a given type of cancer [308, 309]. Considering recent data showing that 2.9 million new cases of cancer were diagnosed in year 2004 and over 1.7 million cancer deaths occurred in Europe alone [310], it is clear that any improvement in predicting chemoresistance or in preventing its arising will have a strong impact on the quality of patient's life and healthcare costs.

CANCER CHEMORESISTANCE: THE RELATIONSHIP BETWEEN N-Myc AND MULTIDRUG TRANSPORTERS IN NEUROBLASTOMA TUMOURS

Although many high-risk neuroblastoma tumours initially respond to the first cycles of intensive chemotherapy, they frequently become refractory to treatment as the disease progresses. Multidrug resistance in neuroblastoma is particularly apparent in patients whose tumours exhibit amplification or over-expression of the *N-MYC* oncogene. *N-MYC* clearly contributes to the drug resistance phenotype of neuroblastoma and it represents one of the most powerful indicators of poor outcome in this disease [69, 70]. However, high levels of *N-MYC* gene expression is strongly predictive of poor prognosis in older children with neuroblastoma, but not in infants [311]. The N-Myc oncoprotein is associated with increased growth potential and tumorigenicity [311] and appears to act as a transcriptional regulator, perhaps influencing the transcription of critical genes involved in multidrug resistance phenomenon, such as ABC transporter genes. Only a single study, performed by *Manohar et al*., demonstrated that N-Myc acts as transcriptional activator on regulation of the *MRP1/ABCC1* promoter in human neuroblastoma [75]. The expression of the *MRP1* gene at high levels in primary neuroblastoma tumours predicts reduced event-free survival and shorter overall survival in children with this neoplasm [72]. Furthermore, since N-Myc regulates expression of *ABCC1,* it has been observed that *N-MYC* amplification or overexpression has no prognostic value when *MRP1* expression is included as a prognostic factor [72].

In this study, we show that several ABC transporters genes, including *ABCC1*, can be regulated by N-Myc, defining the role of the oncoprotein in the development of a multidrug resistance phenotype in neuroblastoma. Indeed, we analyzed the transcriptional profile of all forty-eight human ABC transporter genes in a *N-MYC* inducible neuroblastoma cell line (Tet21/N) and we found that the expression of several ABC genes, such as *ABCA2, ABCB1, ABCB9, ABCB10, ABCC1, ABCC4, ABCE1, ABCF1, ABCF2, ABCF3* and *ABCG2* , paralleled *N-MYC* expression, indicating that a subset of ABC transporter genes can be positively regulated by N-Myc itself. Furthermore, we found an inverse regulation between *N-MYC* and another subset of ABC transporter genes, which seem to be repressed by the oncoprotein. Specifically, for this latter group, we focused on the regulation of the *ABCC3/MRP3* transcription by N-Myc. Indeed, a coordinate expression of *ABCC3/MRP3*, *ABCC1/MRP1* and *ABCC4/MRP4* influences the aggressiveness of neuroblastoma. Multivariate analysis reveals that overexpression of *ABCC1/MRP1* and *ABCC4/MRP4,* when associated with low transcription of *ABCC3/MRP3,* represents the stronger prognostic marker of poor outcome in neuroblastoma tumour (Haber, M. et al. unpublished).

A strong correlation between *N-MYC* expression and transcriptional levels of these ABC drug transporter genes has been demonstrated also in other five neuroblastoma cell lines, which differ from each other to express different levels of N-Myc protein. In particular, ABC genes positively regulated by N-Myc display higher transcriptional activity in neuroblastoma cell lines with *N-MYC* amplification or overexpression than cell lines expressing low levels of the oncoprotein. On the contrary, *ABCC3/MRP3* presents a lower transcriptional activity in those neuroblastoma cell lines with high levels of N-Myc rather than in cell lines where the oncogene is not expressed. This transcription analysis, performed in several

neuroblastoma cell lines as well as in the *N-MYC* inducible system (Tet21/N), allowed us to hypothesize that N-Myc may play a crucial role in the development of multiple drug resistance in neuroblastoma, mediating the transcriptional regulation of several ABC transporter genes.

Furthermore N-Myc, acting as transcriptional regulator of ABC transporter genes, can affect drug resistance in human neuroblastoma. We performed MTT assay to evaluate the survival of different neuroblastoma cell lines after treatment with some chemotherapeutic agents commonly used in therapy, such as vincristine and doxorubicin. We found that neuroblastoma cell lines with amplification or overexpression of *N-MYC* are extremely resistant to chemotherapeutic agents, indicating that N-Myc expression may predict drug resistance due to ABC transporters. Indeed, N-Myc is able to affect drug efflux in human neuroblastoma cancer cells. In the *N-MYC* inducible cellular system, we show that cellular efflux of two dyes, such as Rhodamine123 and DiOC2, is decreased when *N-MYC* is down-regulated. Since these fluorescent molecules are specific substrates of ABCB1/MDR1 and related transporters like ABCC1/MRP1 and ABCG2/BCRP, their decreased efflux, detected in absence of N-Myc, indicate that regulation of ABC transporters by N-Myc itself may affect drug transport outside the cell.

Evaluating the association of the N-Myc/Max heterocomplex with ABC transporter promoters at its specific cognate sites, we can assert that N-Myc can directly regulate transcription of ABC transporters. Formaldehyde ChIP assay performed in Tet21/N cell line reveals that N-Myc is a direct transactivator of many of the ABC genes identified by transcriptional analysis. In agreement with previous reports [229, 312], we observed that most Myc binding sites localize to predicted CpG islands and are proximal to the gene transcription start site, suggesting that the N-Myc/Max heterocomplex may preferentially function close to the transcription start site. We show that, with the exception of *ABCB1/MDR1*, N-Myc/Max is physically associated with the promoter of all transporters upregulated by N-Myc itself, suggesting that N-Myc directly control the transcription of several ABC transporters acting as transactivator factor. Regarding ABCB1/MDR1 we propose that N-Myc regulates its transcription through a secondary mechanism, even if conflicting reports exist regarding the correlation between *MDR1* and *N-MYC*. Indeed, an experimental *in vitro* metastatic neuroblastoma model reported that N-Myc directly regulated *MDR1*gene expression. In this model, the degree of drug resistance directly correlated with the expression levels of *N-MYC* and *MDR1* expression [313]. However, although P-glycoprotein expression was reported to be a predictor marker of outcome in neuroblastoma patients in some studies [71, 314], other series have failed to show any correlation [72, 315]. Furthermore in contrast to *MRP1*, the level of expression of *MDR1* is lower in tumours with *N-MYC* amplification compared to those without amplification [72, 315]. Finally, to confirm that N-Myc binding affects the transcriptional activity of ABC promoters upregulated by N-Myc itself, luciferase assays were performed with a series of ABCs promoter/reporter constructs. Thus, we could recapitulate, in a transient transfection assay, ABC promoters activity as a function of *N-MYC* expression. Furthermore, we can state that the N-Myc binding on tested ABC promoters is required and sufficient to activate their transcription. However, we show that N-Myc may control some ABC transporters transcription also by acting as repressor. In particular, we provide the first evidence to indicate that human *ABCC3/MRP3* is a real N-Myc down-stream regulated gene. c-Myc represses some target genes through three different mechanisms, where c-Myc acts by interacting with other transcription factor, such as Miz1, Sp1 and Smad, and blocking their transacting activity [295, 300]. The mechanisms of repression of c-Myc is different from

that of activation, which is dependent on the direct binding of the Myc/Max heterodimer to the E-Boxes in the target genes. In agreement to this proposition, the N-terminal MBII of c-Myc is required for the repression, but not for activation [230]. Thus, when c-Myc acts as repressor, it binds a DNA promoter region through an interaction with another transcription factor and not directly. Furthermore, in literature there are few studies about c-Myc mediated repression, but there is no evidence regarding N-Myc mediated repression. However, since c- and N-Myc present a significant functional overlapping, we hypothesized that also N-Myc may repress transcription of its target genes by interacting with other factors, such as Miz1or Sp1. *ABCC3/MRP3* promoter contains three Sp1 binding sites (GC box), whereas no E-Box is found in close proximity to the transcriptional start site. We evaluated the binding of N-Myc in the region where GC boxes are located. However, to achieve this aim we set up a new technique, named Dual-Step ChIP. Through this novel approach, we overcame some limits of normal ChIP. In Dual-Step ChIP, to improve the formation of covalent links between proteins and stabilize the association of proteins to DNA, though not directly bound to it, we used cross-linking agents in addition to formaldehyde. Through this technique, we show that either Sp1 or N-Myc binds *ABCC3/MRP3* promoter the +200bp to +500bp region containing multiple Sp1 binding sites. Furthermore, we show, by transient luciferase assay, that this region is strictly required for N-Myc mediated repression of *ABCC3/MRP3* in neuroblastoma. To test that N-Myc really interact with Sp1, we performed a co-immunoprecipitation assay and a GST pull down assay and we established that the central and DNA binding domain of N-Myc are involved in interaction with Sp1. It is clear that the association of N-Myc to *ABCC3/MRP3* core promoter is mediated by Sp1 and in agreement with this speculation, the deletion of the Sp1 binding sites abolished the repression of N-Myc completely. Clinical drug resistance in human neuroblastoma, as in other cancers, is believed to be a multifactorial process involving the action of multiple gene pathways. High-risk neuroblastoma tumours develop resistance phenotype where cancer cells become resistant simultaneously to different drugs with no obvious structural resemblance and with different cellular targets. It is reasonable that several ABC drug transporters with different substrate specificity are involved in the development of this phenotype. In our study, we show that in neuroblastoma cell lines with amplification or overexpression of *N-MYC*, the transcription levels of some ABC genes result increased, influencing the drug sensitivity to some chemotherapeutic agents. Therefore we propose that N-Myc play a key role in the insurgence of drug resistance, because it acts simultaneously regulating the transcriptional levels of several ABC genes. Many ABC transporters upregulated by N-Myc, such as ABCA2, ABCC1, ABCC4, ABCG2 pumps drug molecules from the cytosol to the extracellular medium. However, intracellular drug concentrations often remain high in drug resistance cells and therefore do not explain how drug pumping at the plasma membrane confers multidrug resistance. Recent work indicates how drug sequestration in cytoplasmic organelles can account for these paradoxical results. The intracellular drug distribution is modified in many MDR cell lines, leading to increased drug sequestration in acidic vesicles, such as the *trans*-Golgi apparatus, recycling endosomes, and lysosomes, followed by transport to the plasma membrane and extrusion into the external medium. Since most anticancer agents target DNA or nuclear enzymes, sequestration of drug in cytoplasmic organelles will lead to decreased drug-target interaction and thereby, decreased cytotoxicity [316, 317]. Some ABC transporters up-regulated by N-Myc, such as ABCB9 and ABCB10, are expressed in mitochondria and lysosomes respectively and may contribute to alter intracellular drug concentration. N-Myc controls the development of

chemoresistance phenomenon mainly acting as transactivator factor. However, we provide the evidence that *ABCC3/MRP3* is repressed by N-Myc. Although, MRP3 is thought to increase elimination of bile salts from the hepatocyte, it is expressed also in normal human adrenal gland, in the cells of the zona fasciculata and in the zona reticularis [38]. Most neuroblastomas arise from the adrenal glands and may develop anywhere along the sympathetic chain [318]. Thus, we hypothesize that *MRP3* may be silenced during the development of neuroblastoma tumour at the same time with *N-MYC* amplification or overexpression. Furthermore, non steroidal anti-inflammatory drugs induce *MRP3* expression in colorectal cancer and seems to be involved in the suppression of tumorigenesis [319]. Initial studies on MRP3 in a panel of drug-resistant cancer cell lines did not turn up any association between MRP3 levels and resistance [37] and clinical studies have revealed that low expression of *ABCC3*, when associated with high transcriptional levels of *ABCC1* and *ABCC4*, is indicative of poor outcome in primary human neuroblastoma (Haber, M. data unpublished). These evidences support the hypothesis that *ABCC3* may act as tumor suppressor gene and for this reason it might be necessary to silence its expression during tumour development.

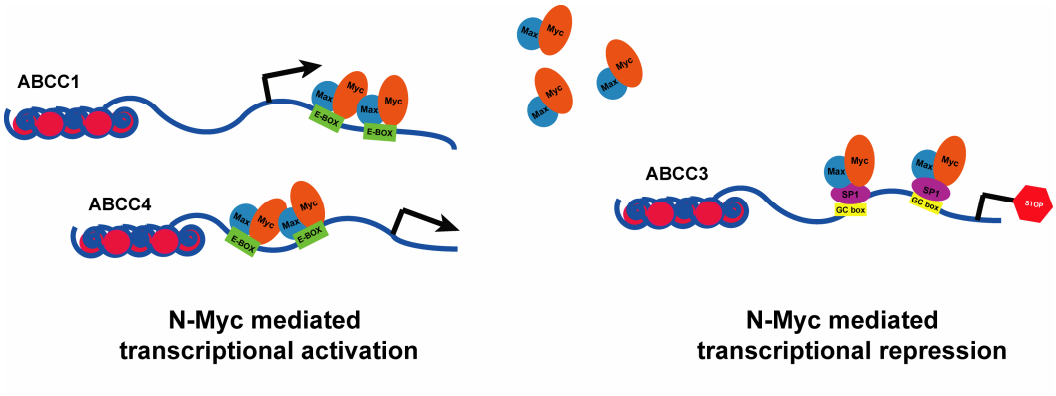


Figure 1. Coordinate regulation of ABC drug transporter genes by N-Myc in human neuroblastoma cell lines.

Taken together, this study indicates that N-Myc plays a key role in controlling the development of drug resistance phenotype in human neuroblastoma, operating a direct and concerted regulation of multidrug resistance genes, acting either as transactivator or as transcriptional repressor (Fig.1). For these reasons, strategies aimed at inhibiting *N-MYC* may have therapeutic potential in children with aggressive neuroblastoma, above all N-Myc may represent a downstream target to control the insurgence of drug resistance.

CANCER CHEMORESISTANCE: THE RELATIONSHIP BETWEEN c-Myc, MULTIDRUG TRANSPORTERS AND EPIGENETIC EVENTS.

Our study propose that, like N-Myc, also c-Myc plays a crucial role in the development of drug resistance phenomenon in leukaemia cell lines, such as lymphoblastoid and promyelocytic cell lines. *c-MYC* overexpression has been demonstrated in many human malignancies and it is involved in the neoplastic transformation process. So far, no lines of evidence have been provided about its ability to control the drug resistance in cancer. According to our findings c-Myc can control transcriptional activity of several ABC drug transporters, by directly binding their promoters at its cognate sites. Like N-Myc, c-Myc may induce an

overexpression of ABC transporters, such as ABCA2, ABCC1, ABCC4, involved in the pumping of drug molecules from the cytosol to the extracellular medium. However, like N-Myc, also c-Myc is involved in the intracellular drug distribution, controlling transcriptional levels of ABC transporters, such as ABCB9, ABCB10, localized in cytoplasmic organelles. Therefore, c-Myc may regulate drug concentration in tumor cells increasing drug efflux of tumour cell and increasing drug sequestration in acidic vesicles, such as *trans*-Golgi apparatus, leading to decreased drug-target interaction and thereby decreased cytotoxicity. Altered intracellular drug distribution and the overexpression of drug efflux pumps are usually associated in cancer cells which develop drug resistance.

Finally, either N-Myc or c-Myc are direct transactivator of *ABCE1* transporter gene. Although this gene is a member of the ATP-binding cassette (ABC) multigene family, it is mainly involved in the inhibition of ribonuclease L, a nuclease induced by interferon in mammalian cells. Human ABCE1 interacts also with the eukaryotic initiation factors eIF5 and eIF2 components of the pre-initiation complex[46], thereby Myc proteins, inducing *ABCE1* overexpression, control translational process, determining a dramatic increase of cellular growth.

It is interestingly to note that in leukaemia cell lines, c-Myc is not able to control transcriptional levels of *ABCB1* and *ABCG2*. Both these genes seem to be silenced in some leukaemia cell lines. Regarding *ABCB1* some studies implicated epigenetics in the control of its transcription [320]. The *ABCB1/MDR1* promoter region, including exon 1 and intron 1, is GC rich and constitutes a CpG island according to specified criteria. Several studies demonstrate that methylation of the *ABCB1/MDR1* promoter is linked with a lack of *MDR1* expression in cell lines and samples from patients that were diagnosed with AML, acute lymphocytic leukaemia, chronic lymphocytic leukaemia, colorectal cancer and bladder cancer. Our findings demonstrate that also *ABCG2* promoter is methylated in leukemia cell lines, even if Myc binding site continue to be unmethylated, indicating that other heterochromatic markers may avoid the binding of c-Myc on the *ABCG2* promoter. Furthermore, we demonstrate that treatment with 5'-Aza-2'-deoxycytine results in a activation of *ABCG2* expression, as well as other ABC transporter genes. Therefore, epigenetic events play a crucial role in controlling ABC transporter gene expression. Recently, the 5'-Aza-2'-deoxycytine (decitabine) has been approved for the treatment of myelodisplastic syndromes and other kind of leukaemias. Decitabine is a hypomethylating agent and at low concentrations is considered to exert its anticancer effects by inducing hypomethylation of tumour suppressor genes [321]. However we propose that its effects may be broader and may lead to reactivation of other genes, such as some ABC drug transporters, promoting the development of drug resistance phenomenon and increasing the aggressiveness of the tumour. Finally, the treatment with demethylating agents may play a critical role in misregulating Myc transcription functions during tumour development, allowing the binding of the oncoprotein at the promoters, such as *ABCG2* promoter, and causing altered expression of its target gene, otherwise silenced. Interestingly, hypomethylation correlates also with overexpression of *c-MYC* and an increased frequency of hematological tumours in mice [322]. For these reasons, the therapy with demethylating agents could result ineffective and dangerous in treatment of cancer.

CANCER CHEMORESISTANCE: THE ROLE OF c-Myc IN CONTROLLING EXPRESSION OF MDR IN HEMATOPOIETIC CANCER STEM CELLS.

Recent findings have demonstrated that cells with stem-cell qualities are present in malignancies of haematopoietic origin and in some solid tumours. The existence of such population would imply that the stem cell represents the cell of origin for the tumour. Cancer stem cells are likely to share many of the properties of normal stem cells that provide for a long lifespan, including relative quiescence, resistance to drugs and toxins through the expression of several ABC transporters, an active DNA-repair capacity and a resistance to apoptosis [79]. This observation supports a novel point of view on how drug resistance may be achieved by cancer cells. In the conventional model of tumour cell drug resistance, rare cells with genetic alterations that confer multidrug resistance (MDR) form a drug resistant clone. Following chemotherapy, these cells survive and proliferate, to form a recurrent tumour that is composed of offspring of the drug resistant clone. In the alternative model, drug resistance can be mediated by stem cells. According to this model, tumours contain a small population of tumour stem cells and their differentiated offspring, which are committed to a particular lineage. Following chemotherapy, the committed cells are killed, but the stem cells, which express drug transporters, survive. These cells repopulate the tumour, resulting in a heterogeneous tumour composed of stem cells and committed but variably differentiated offspring [79]. The drug transporting property of normal and cancer stem cells conferred by ABC drug transporters represents an important marker in the isolation and analysis of haematopoietic stem cells. Most cells accumulate the fluorescent dyes Hoechst 33342 and Rhodamine 123, but stem cells do not, as these compounds are effluxed by ABCG2/BCRP and ABCB1/MDR1, respectively. Because they don't accumulate these fluorescent dyes, stem cells can be sorted by collecting cells that contain only a low level of Hoechst 33342 fluorescence. These cells are referred to as "side population" (SP) cells [79]. A large fraction of haematopoietic stem cells are found in the SP fraction [105]. The exact origin of pluripotent stem cells in tumours might vary. They could arise from the malignant transformation of a normal stem cell that has accumulated oncogenic insults over time [79]. Alternatively, the original tumour cell could be a more differentiated cell that develops the capacity for continual self-renewal, thus acquiring the properties of a stem cell [323]. Distinguishing between these two might be difficult. Evidence that cells other than stem cells can acquire the ability to undergo self-renewal has been recently proposed in studies examining the progression of chronic myelogenous leukaemia (CML) [83]. The chronic phase of this disease occurs when a stem cell acquires the expression of BCR-ABL fusion protein, leading to increased proliferation of cells within the granulocyte-macrophage progenitor pool and their downstream progeny. Furthermore, the progression to blast crisis follows additional genetic or epigenetic events that confer progenitor stem cells with the capacity to self renew, making them indistinguishable from a leukaemic stem cell. For these reasons, the stem cell compartment is not rigidly defined, suggesting a degree of plasticity in CML, and generally in cancer [79, 83]. It was therefore important to identify the population that contains leukemic stem cells in CML and to identify events leading to the progression of leukaemia, the outcome of these events, and the order of their appearance in leukemic stem cells and their precursors.

In our study, we isolated a stem cell population from newly diagnosed chronic phase (CP)-CML patients. Indeed, CD34 is a glycophosphoprotein expressed on early hematopoietic precursor cells, which can be easily identified and purified by immunological tecniques. Immunophenotype of bone marrow (FACS analysis) in

CML patients revealed a broader CD34+ cell population than that revealed in healthy donors. The number of CD34+ progenitor cells provides useful diagnostic and prognostic information regarding the evolution of CML. Furthermore, in chronic myelogenous leukaemia, cancer stem cells CD34+ are characterized by the t(9;22) (q34;q11) reciprocal chromosomal translocation (chromosome Ph), which forms the *BCR-ABL* fusion gene [324]. We find that these cancer stem cell population expresses high transcriptional levels of several ABC drug transporter genes if compared to those observed in the entire population of mononuclear tumour cells from which CD34+ stem cells have been purified. We show that also *c-MYC* is highly expressed in CD34+ stem cells fraction and that it is almost silenced in mononuclear tumor cells. Therefore, we can hypothesize that, in CML, CD34+ cancer stem cells are mainly responsible to develop aggressive forms of this kind of leukaemia, and that, expressing high levels of several drug transporters, they may repopulate the tumour after chemotherapy, spawning a tumour mass completely refractory to treatment with chemotherapeutic agents. It is interesting to point out that in patients with a response to imatinib there is a significant decrease in the number of CD34+ cells, whereas samples from patients with imatinib-resistant CML increase number of granulocyte-macrophage progenitors [83]. Resistance to imatinib is generally seen in patients who display a genomic amplification of *BCR-ABL* or point mutations in the BCR-ABL kinase domain. However, resistance to imatinib may be due to overexpression of some ABC transporters [325]. Indeed, imatinib is a good substrate for the ABCG2 drug pump [325], whose expression is regulated by c-Myc. Furthermore, *ABCG2* promoter is often silenced in leukaemia. However, we show that CD34+ cancer stem cell population derived from CML patients expresses *ABCG2/BCRP*. This evidence support the model for which high-expression/hypomethylated *ABCG2* cells has an advantage during chemotherapy.

In Kazumi- 4, a cell line derived from a Ph+ CML patient and that expresses the CD34 antigen, we show that c-Myc is directly associated with the promoter of ABC genes highly expressed in CD34+ cancer stem cells. Taken together, our findings support the hypothesis that c-Myc, acting as a direct transactivator of several drug transporters, play a key role in controlling the development of drug resistance in cancer stem cells. In CML, it is also known that *c-MYC* expression is required for the oncogenic effects of BCR-ABL and that the overexpression of *c-MYC* is mediated by BCR-ABL itself, through the Jak2 kinase [304, 326]. How c-Myc might do in CD34+ Ph+ cells was still unknown. In our study we propose that one possible effect of c-Myc in CD34+ Ph+ cells is linked to its capacity to develop drug resistance, by inducing transcriptional activity of some ABC drug transporters. Therefore, through this mechanism, c-Myc may mediate the outward-directed transport of imatinib, determining the failure of chemotherapeutic treatment in CML.

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