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Promiscuity of Translocation Partners in Multiple Myeloma

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ABSTRACT

Multiple myeloma (MM) is characterized by karyotypic instability, including chromosomal translocations involving the *IGH* locus. MM cells display a promiscuity of translocation partners, only some of which are recurrent. We propose that several factors, including temporal and spatial nuclear positioning of potential partner loci, "off-target" *IGH* diversification mechanisms, and aberrant repair pathways contribute to the promiscuity of translocation partners in MM. We speculate that in MM, *IGH* diversification processes [V(D)J recombination, somatic hypermutation, and class switch recombination] in B cells may not be restricted to specific stages of B-cell development or within specific immune tissues, but may occur in different temporal "windows." Before or during MM evolution, off-target activities of the enzymes involved in *IGH* modification processes may contribute to the generation of double-strand breaks (DSB) in translocation partner loci. In the parent B cells from which MM originates, spatial proximity within the nucleus of *IGH* and potential translocation partners contributes to the selection of a translocation partner and the clinical frequency at which a specific translocation occurs. The spatial proximity of *IGH* and specific translocation partners may be temporal and contribute not only to partner selection but also to the promiscuity of partners seen in MM. Lastly, aberrant repair mechanisms in MM progenitors (including the possibility that a Ku 86 variant allows for positional instability at DSBs) may also contribute to the promiscuity of chromosome translocation partners in MM. J. Cell. Biochem. 109: 1085–1094, 2010.

KEY WORDS: MULTIPLE MYELOMA; CHROMOSOME TRANSLOCATION; CHROMOSOME POSITIONING; *IGH* DIVERSIFICATION; Ku86

Multiple myeloma (MM) is a fatal, malignant tumor of B-lineage hematopoietic cells; the pathology mediated by an accumulation of malignant plasma cells (PCs) in the bone marrow (BM). This abnormal infiltration of PCs in the BM results in the production of monoclonal immunoglobulin (Ig) that is detectable in the serum and urine and can lead to renal failure. Cells of the MM tumor clone can be uniquely identified by a heavily mutated, class-switched clonal Ig heavy chain (*IGH*) gene rearrangement, which provides a molecular signature. A considerable body of evidence implicates B cells as generative compartments of the MM clone; including molecular analysis which suggests that the malignant clone in MM arises from a memory B lymphocyte. The involvement of B-cell progenitors and earlier stage cells in MM has been documented by molecular, phenotypic, and functional studies, with the rearranged *IGH* variable, diversity, and joining [V(D)J] genes providing a unique clonal marker to identify cells within the myeloma clone [Bergsagel et al., 1995; Szczepek et al., 1998; Reiman et al., 2001; Pilarski and Belch, 2002; Adamia et al., 2005; Kirshner et al., 2008; Pilarski et al., 2008]. MM is characterized by multiple chromosomal abnormalities, with extensive heterogeneity among PCs from the same patient and among different patients. This instability may be a consequence of abnormal mitoses that result in chromosomal mis-segregation, such as that mediated by over-expression of RHAMM [Maxwell et al., 2003, 2005].

The replacement of normal BM cells by malignant PCs results in anemia, cytopenia, and subsequent immune failure. Hypercalcemia, osteoporosis, fractures, spinal cord compression, and painful osteolytic bone lesions are direct consequences of the interaction of malignant PCs with the BM microenvironment [reviewed by Sirohi and Powles, 2004]. Front-line treatment of MM involves chemotherapy followed by autologous stem cell transplant, but despite various treatment regimens and development of new chemotherapeutic agents, there is currently no cure, and patients ultimately succumb to this devastating disease.

The characteristic karyotypic instability seen in myeloma includes chromosomal translocations. Chromosomal translocations in MM most frequently involve the *IGH* locus at 14q32, as with many B-cell malignancies [Fabris et al., 2005; reviewed by Liebisch and Dohner, 2006]. Translocations involving the *IGH* locus are present in 60% of the PCs from myeloma patients, and in 90% of human myeloma cell lines (HMCLs), suggesting that the prevalence of *IGH* translocations may increase with disease progression. The

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translocations are mediated by errors in *IGH* modification processes necessary for generating antibody diversity. An overview of those processes will be outlined below.

DIVERSIFICATION OF THE *IGH* LOCUS

The *IGH* locus at 14q32 is composed of 44 functional variable (V_H), 27 diversity (D_H), 6 joining (J_H), and 8 constant (C_H) gene segments [Matsuda et al., 1998]. To generate the antibody diversity necessary for the unique specificity of the humoral response, the *IGH* locus in B cells undergoes three DNA modification processes during B-cell development: (1) variable–diversity–joining [V(D)J] recombination; (2) *IGH* class switch recombination (CSR); and (3) somatic hypermutation (SHM). All three processes have the potential to produce double-strand breaks (DSBs) within the *IGH* locus.

V(D)J RECOMBINATION

Prior to antigen exposure, developing B cells in the BM undergo germline V(D)J recombination of the *IGH* which results in the production of a primary arsenal of antibody specificities. The process is initiated by the recombinase-activating gene (RAG)-1 and -2 proteins [reviewed by Fugmann et al., 2000]. These two proteins form an endonuclease complex which induces site-directed DSBs at recombination signal sequences (RSS) flanking each gene segment within the V_H, D_H, and J_H regions of *IGH*, and the V_L and J_L regions of the Ig light chain *IGL*. Within the *IGH* locus, the D_H to J_H regions recombine first, followed by the V_H to DJ_H. After recombination, the DSBs are rejoined by the non-homologous end-joining pathway (NHEJ), which ligates DNA ends with little or no stretches of homology. The resulting IgM antibodies are of low affinity but high avidity, providing a first-line adaptive defense against pathogens and their products. B cells can also undergo a second round of *IGL* recombination at later stages. This recombination is termed "receptor editing" and its purpose is to ablate autoreactivity.

SOMATIC HYPERMUTATION

Once stimulated by antigen, the mature B cells are directed to proliferate, differentiate, and migrate to the germinal centers (GCs) of the secondary lymphoid organs, such as lymph nodes, tonsils, and spleen [Kelsoe, 1996]. In GCs, the activated B cells undergo SHM of the VDJ_H region. By mechanisms that are not fully understood, it is believed that SHM is generated either by direct replication or by error-prone repair systems resolving VDJ_H region DNA lesions [including DSBs, Bross et al., 2000; Papavasiliou and Schatz, 2000], triggered directly or indirectly by the enzyme, activation-induced cytidine deaminase (AID). During high rates of transcription, AID deaminates cytosine residues to generate U:G mismatches on single-stranded DNA [Peters and Storb, 1996; Fukita et al., 1998]. The resulting point mutations create changes in the coding sequence necessary for affinity maturation and antibody specificity [reviewed by Di Noia and Neuberger, 2007]. The variety of base substitutions generated is dependent upon the normally error-free pathways used to process the U:G lesions: (1) general replication, (2) uracil DNA glycosylase (UNG) followed by base excision repair (BER), or (3) mismatch repair (MMR). Somehow, these repair pathways are redirected from their normal roles of preserving genome integrity to processing U:G mismatches in an error-prone manner to generate the *IGV* diversification necessary for the humoral response [reviewed by Peled et al., 2008]. It is interesting to note that VDJ_H rearrangements in myeloma contain higher mutation rates than any other B-cell malignancy [reviewed by Gonzalez et al., 2007].

CLASS SWITCH RECOMBINATION

Activated B cells also undergo CSR in GCs. This process is required to change the effector functions of antibodies involved in the humoral response [reviewed by Chaudhuri et al., 2007]. The process of CSR exchanges the exon coding for the μ constant domain of *IGH* with one of the downstream exons coding for α, δ, ε, γ1, γ2a, γ2b, or γ3 [Honjo et al., 1981]. Directed DSBs are produced through the action of AID at specialized switch (S) regions located upstream of the exons encoding the various constant regions of *IGH*. Switch regions consist of tandem repeats that are unique to each isotype, although all contain the "hotspot" motif WRC/GYW, where W = A or T, R = G or A, and Y = C or T [Schrader et al., 2007]. During switch region transcription, the enzyme deaminates cytosine residues on single-stranded DNA to produce U:G mismatches. The mismatches are converted into abasic sites, through uracil excision by UNG. Apurinic/apyrimidinic endonuclease 1 (APE-1) nicks the phosphodiester backbone at the abasic site [Rada et al., 2004]. CSR works on both transient single strands exposed during transcription, resulting in DSBs. AID-dependent DSBs are introduced and repaired in G1 [Schrader et al., 2007]. As for V(D)J recombination, the DSBs are repaired by the NHEJ pathway, as the nature of S region sequences (lack of long stretches of perfect homology between switch regions) would not support homologous recombination (HR) [reviewed by Kotnis et al., 2009]. The result is a hypermutated, function-specific antibody. Myeloma displays the following distribution for *IGH* expression: 60% IgG, 24% IgA, 3% IgD, and 2% biclonal or other isotypes, including IgE and IgM. The remaining 11% of myelomas produce light chains only [reviewed by Gonzalez et al., 2007]. The presence of a single, unchanging clonotypic switch junction in MM PCs suggests that myeloma progenitors reside in the post-switch population [Taylor et al., 2008].

CHROMOSOMAL TRANSLOCATIONS INVOLVING THE *IGH* LOCUS IN MM

The effect of the resulting translocation is likely to be deregulation (increased expression in the case of MM) of an oncogene, as it is repositioned near one or more of the strong *IGH* enhancers [reviewed by Kuehl and Bergsagel, 2002]. Whereas many other B-cell malignancies harbor a single, specific *IGH* translocation partner [reviewed by Kuppers, 2005], myeloma cells display promiscuity of translocation partners. There are five primary *recurrent* partner loci harboring the following oncogenes, with the indicated approximate frequencies:

- 4p16 (*MMSET* and usually *FGFR3*)—15%.
- 6p21 (*CCND3*)—3%.
- 11q13 (*CCND1*)—20%.

- 16q23 (*c-MAF*)—5%.
- 20q12 (*MAF-B*)—2%.

What is the reason for partner promiscuity seen in MM? And why is a specific partner preferentially selected for translocation?

The answers to these questions are currently not known. What is known, however, is that for chromosomal translocations to occur, two prerequisites are necessary; (1) the two potential chromosome partners must be in close proximity and (2) simultaneous DSBs on each potential chromosome partner must occur. Several studies elucidating these mechanisms may provide insight into the above questions.

GENOME POSITIONING IN THE INTERPHASE NUCLEUS

Fluorescence in situ hybridization (FISH) studies have established the existence of unique chromosomal territories (CTs) in the interphase nuclei of higher organisms [Schardin et al., 1985; Cremer and Cremer, 2001; Parada and Misteli, 2002]. The spatial organization of both chromosomes and genetic loci is not random and can be described by their probabilistic radial positioning relative to the center of the nucleus [Cremer and Cremer, 2001; Parada and Misteli, 2002; Parada et al., 2003; Taslerova et al., 2006]. It is generally assumed that the periphery of the nucleus is a transcriptionally inactive area; conversely, the interior of the nucleus is a transcriptionally active area by [reviewed by Lanctot et al., 2007].

NON-RANDOM SPATIAL POSITIONING OF CHROMOSOMES

The radial CT positions within the interphase nucleus correlate with gene density as well as with chromosome size. In human fibroblasts, large chromosomes preferentially localize to the nuclear periphery, while smaller chromosomes localize more centrally, which is not as pronounced in non-adherent cells [Mayer et al., 2005]. In both cell types, gene dense chromosomes adopt a more central location in the cell nucleus, while gene poor chromosomes are more peripherally located [Murmann et al., 2005]. In addition, gene dense and gene poor regions *within* individual chromosomes cluster to specific areas within the CT boundary, with gene dense areas closer to the nuclear center and gene poor areas closer to the periphery [Croft et al., 1999; Cremer et al., 2003]. Radial CT positioning according to gene density is evolutionarily conserved in primates [Tanabe et al., 2002, 2005], mice [Mayer et al., 2005], and chickens [Habermann et al., 2001], suggesting a functional role for positioning in vertebrates [Parada and Misteli, 2002].

Chromosome positioning is not exclusively characterized by gene density and chromosome size, as positioning can be variable. Proliferation [Mehta et al., 2007; Meaburn et al., 2007a], differentiation [Kim et al., 2004; Stadler et al., 2004; Mayer et al., 2005], and tissue specificity [Parada et al., 2004a; Mayer et al., 2005] have each been shown to influence CT positioning. While gene density and chromosome size remain the same, these examples vary with regard to their gene expression profiles, further suggesting that functional status of each chromosome also plays a part in its positioning [Meaburn et al., 2007b]. The obvious function would be control of gene expression [reviewed by Guasconi et al., 2005].

NON-RANDOM SPATIAL POSITIONING OF GENES

Genes are also non-randomly positioned within the nucleus during interphase [Roix et al., 2003; Misteli, 2005]. The localization of various genes is not static and is dependent upon activity and level of gene expression. Generally, transcriptionally active genes are more internally localized, while inactive genes are more externally located [reviewed by Williams et al., 2006; Meaburn et al., 2007b]. For example, *IGH* and *CD4* localize internally in their active state, and to the nuclear periphery in their inactive state [Skok et al., 2001; Kosak et al., 2002; Kim et al., 2004]. *IGH* allelic exclusion, which takes place during B-cell development, results in the unrearranged allele moving to an area of centromeric heterochromatin [Roldan et al., 2005]. In resting splenic B cells, mono-allelic recruitment to centromeric heterochromatin also takes place, and the allele is transcriptionally silenced [Skok et al., 2001].

In addition to possessing preferred positions within the nucleus, genes also localize to favored locations within their respective CT. Genes have been shown to "loop out" of their respective chromosome territory, correlating with local gene density and transcription [Mahy et al., 2002; Williams et al., 2002]. It has been postulated that specific positioning of a gene in the cell nucleus is not essential to its function but contributes to optimizing its activity [reviewed by Meaburn and Misteli, 2007].

RELATIVE SPATIAL POSITIONING OF CHROMOSOMES AND GENOMIC SEQUENCES

Subsets of specific chromosomes have cell-type specific preferred positioning relative to one another in the interphase nucleus in mouse [reviewed by Parada et al., 2002, 2004b], and in human cells [Bartova et al., 2002]. Specific genomic sequences (genetic loci or gene regulatory regions) also maintain preferred relative positioning. Exciting observations suggest that genetic loci interact with distal regulatory regions in *cis* (same chromosome) [Osborne et al., 2004], or in *trans* (different chromosome) [Spilianakis et al., 2005; Brown et al., 2006; Ling et al., 2006; Lomvardas et al., 2006]. These physical interactions are a remarkable example of regulated 3-D genome architecture and have important functional consequences as regulatory regions are brought into proximity with otherwise distant genes to regulate their expression [Spilianakis et al., 2005; reviewed by Meaburn and Misteli, 2007].

TRANSLOCATION BREAKPOINTS WITHIN THE *IGH* LOCUS IN MYELOMA

During B-cell development, allelic exclusion of *IGH* ensures the expression of a single antibody by a given cell. Despite the likelihood that recombination machinery acts equally on both alleles, it appears that translocations involving switch regions occur preferentially with the excluded IgH allele, based on the fact that only a small percentage of myelomas are non-secretory. This

observation has not been adequately explained but may involve the relocalization of the excluded allele to repressive subnuclear compartments (such as centromeric heterochromatin clusters or the nuclear periphery) [Roldan et al., 2005] where preferential proximity to potential translocation partners may occur. Most translocations in myeloma appear to involve DSBs located in or near the switch regions. It has been shown, however, that most studies detecting and cloning breakpoints in myeloma have relied on molecular methods, which significantly bias the results toward identifying translocations which occur in or near switch region. Translocations involving breakpoints in the J_H region have been reported in patients displaying t(4;14), and in as many as 50% of the t(11;14) [reviewed by Gabrea et al., 2006], suggesting a recombination event outside the switch region. The location of a breakpoint in the *IGH* locus has functional implications for the predicted expression of oncogenes on the derivative chromosomes (Fig. 1). V(D)J recombination and SHM generate breakpoints upstream of Eμ, maintaining Eμ and the Eα enhancers on der(14). CSR mediates breakage in or near switch regions and causes segregation of the Eα enhancers on der(14), but Eμ is recruited to the other derivative chromosome. The former break can dysregulate a single oncogene on der(14) alone, whereas the latter break has the potential to upregulate two oncogenes; one on each derivative chromosome. The breakpoints in the three most common translocations in myeloma will be discussed.

t(4;14)

The translocation event involved in t(4;14) involves 14q32 and 4p16, and displays a prevalence of ~15% among myeloma patients [Keats et al., 2003, 2006]. The t(4;14) is karyotypically cryptic; however, the breakpoints in this translocation have been heavily documented through the identification of illegitimate switch recombination fragments. The majority of t(4;14) breakpoints recorded thus far in tumors or HMCLs involve switch regions [reviewed by Chesi et al., 1998b; Bergsagel and Kuehl, 2001; Fenton et al., 2003; Keats et al., 2003]. Approximately two-thirds of the 5′ switch breakpoints on der(4) involve Sμ, and the other third involve recombined hybrid Sμ/Sγ or Sμ/Sα regions. The 3′ switch breakpoints on der(14) utilize Sμ, Sγ, or Sα regions with similar frequencies, and occasionally hybrid switch regions (Sμ/Sγ, Sμ/Sα, Sα/Sγ) [reviewed by Gabrea et al., 2006]. There is one report of an IgM myeloma case with t(4;14) [Ackroyd et al., 2005].

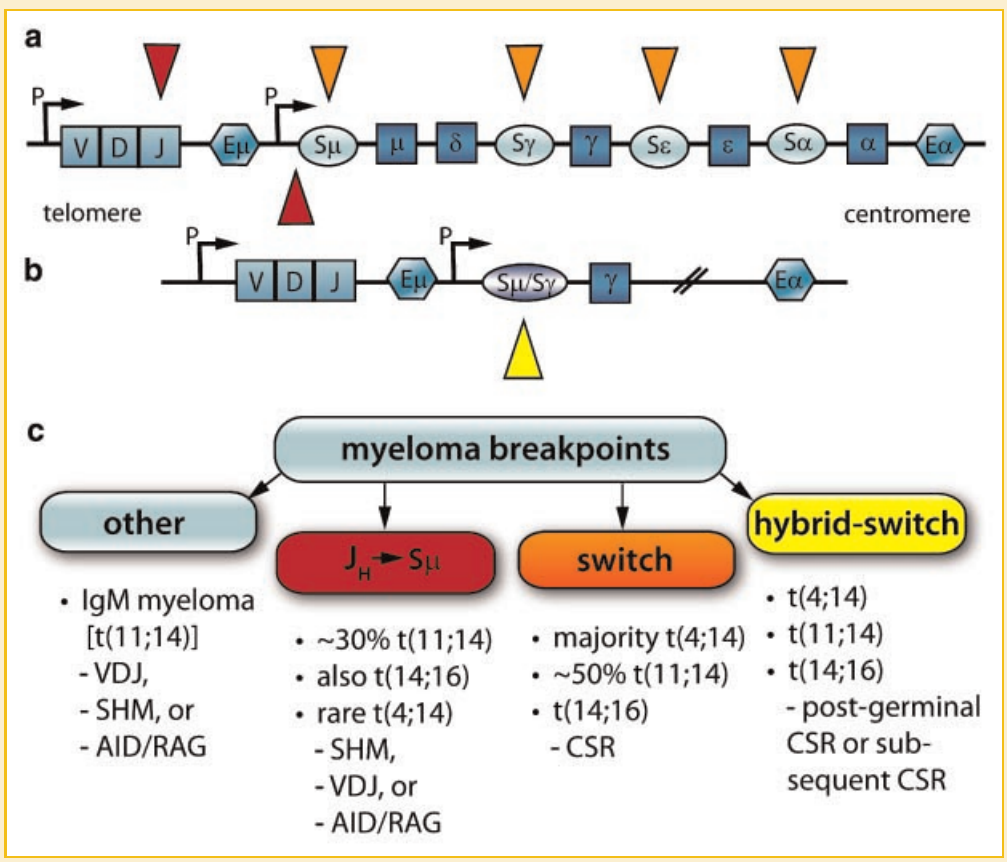

Fig. 1. Possible aberrant recombination events responsible for primary translocations in multiple myeloma. a: The clonal VDJ pre-switch IgH locus at 14q32. Breakpoints mediated by aberrant VDJ recombination or SHM occur centromeric to J_H and telomeric to Sμ. The region is indicated by red arrowheads. Recent data indicate that these breakpoints may be mediated by combination enzymatic activity of AID/RAG (see text). Breakpoints mediated by CSR occur within or near switch regions and are indicated by orange arrowheads. b: The clonal VDJ post-switch IgH locus at 14q32. Breakpoints mediated by post-switch or subsequent post-germinal CSR occur in hybrid switch regions and are indicated by yellow arrowheads. c: Summary of myeloma breakpoints, and involved translocations and possible aberrant mechanisms.

t(11;14)

The translocation event involved in t(11;14) involves 14q32 and 11q13 and displays a prevalence of ~20% among myeloma patients. In contrast to t(4;14), aberrant CSR is responsible for only 50% of t(11;14) in myeloma [reviewed by Gabrea et al., 2006]. Some of these events show breakpoints involving recombined hybrid switch region sequences, as DNA from chromosome band 11q13 has been shown to be joined to (Sμ/Sγ) in myeloma patients [Fenton et al., 2004]. This suggests that this translocation event is more complex than canonical CSR, providing evidence that a translocation event occurred post-switch, or due to successive rounds of CSR.

The remaining translocations involve breakpoints in J_{H5}, J_{H6}, or the region upstream of Sμ, and may be the result of aberrant V(D)J recombination or SHM [Janssen et al., 2000; Fenton et al., 2004]. Several studies of IgM myelomas demonstrate that t(11;14) is overrepresented in this group. In a collection of 33 IgE, IgD, IgM, and non-secretory myeloma patients, 83% displayed t(11;14). Seven of the eight IgM myeloma patients characterized were identified as t(11;14). The IgG and IgA control population of myeloma patients had an incidence of 15% and 10%, respectively [Avet-Loiseau et al., 2003a]. A similar, more recent study of 10 patients with IgM myeloma showed that 5/8 cases assessed had t(11;14). Interestingly, this translocation has not been found in IgM malignancies classified as Waldenstrom's macroglobulinemia [Avet-Loiseau et al., 2003b].

t(14;16)

The translocation event involved in t(14;16) involves 14q32 and 16q23 and displays a prevalence of ~5% among myeloma patients [Bergsagel and Kuehl, 2005]. Partly due to the low clinical frequency, much less information is available regarding the *IGH* breakpoints in this translocation type. In a study of five HMCLs with t(14;16), two 5′ switch breakpoints on der(16) mapped to Sμ. Of the four 3′ switch breakpoints identified on der(14), two mapped to Sμ, one to Sγ, and one Sμ/Sγ. The fifth cell line displayed a der(14) breakpoint located near J_{H5} [reviewed by Chesi et al., 1998a; Bergsagel and Kuehl, 2001].

The above processes provide a feasible model to explain the aberrant recombination mechanisms involved in creating DSBs in the *IGH* locus (see Fig. 1), but for translocations to occur, DSBs must also be generated on the potential partner chromosome as well. How is this achieved?

OFF-TARGET ACTIVITIES OF AID AND RAG

It has been known for some time that in diffuse large cell lymphoma, aberrant SHM results in frequent mutation of the genes *Pax*, *C-myc*, *Rho/TTF*, and *Pim-1*. Interestingly, each of these four oncogenes is also involved in chromosomal translocations with breakpoints situated within the mutated regions, indicating the involvement of "off-target" AID activity in the generation of translocations [Pasqualucci et al., 2001]. Recently, Liu et al. [2008] demonstrated that in addition to the above-listed genes, AID has the potential to trigger mutations in numerous other tumor-related genes, including *H2AX*, *Ocab*, and *Ebf1* in *normal* B cells; indicating that the mutations may be a result of the breakdown of high-fidelity repair during B-cell transformation. Robbiani et al. [2008] demonstrate that AID is essential for the DSBs that form in *c-myc*/*IGH* translocations seen in activated B cells, and that this activity is dependent upon a functional *c-myc* and *IGH* promoter [Robbiani et al., 2008]. The rate-limiting step in the formation of translocations is the generation of DSBs in *c-myc* (which is lower than in *IGH*) and appears to be the result of errors in the otherwise relatively error-free repair of AID lesions in *c-myc*. This suggests that a faulty repair mechanism may also be responsible for the breakage at *c-myc*.

Further studies demonstrate that both RAG and AID, either alone or in concert are also capable of "off-target activities" involving oncogenes. In an effort to understand the mechanisms involving DSBs in *bcl-2*, *bcl-1* (*CCND1*), and *E2A*, Tsai et al. analyzed 1,700 breakpoints known to occur in human lymphomas and determined that a large proportion of the breakpoints are enriched in CpG dinucleotides. They show that T:G mismatches can generate a DNA structure that is recognized as a substrate by the RAG endonuclease complex. Since the cytosine of the CpG dinucleotide is a target for methylation, resulting in the conversion of cytosine to thymine, they theorize that AID may modify methylated CpG dinucleotides resulting in a T:G mismatches which are acted upon by RAG. This theory relies on the tenet that B cells can express both RAG and AID simultaneously. Of interest, both aberrant BER and MMR during SHM can result in T:G mismatches [reviewed by Peled et al., 2008].

BREAKPOINTS MAPPED TO 4p16

The 4p16 breakpoints seen in t(4;14) are located at several sites near or within the MMSET gene, approximately 80–150 kb centromeric to the 5′ end of the FGFR3 gene [reviewed by Chesi et al., 1998b; Bergsagel and Kuehl, 2001; Fenton et al., 2003; Keats et al., 2003]. Several breakpoints appear to cluster in region surrounding exon 1, and introns 3 and 4 of the MMSET gene; however, the significance of these breakpoint clusters is not known [Keats et al., 2005; reviewed by Gabrea et al., 2006]. The apparently exclusive identification of CSR *IGH* breakpoints in t(4;14) suggests that off-target CSR is responsible for the generation of DSBs at 4p16.

BREAKPOINTS MAPPED TO 11q13

The 11q13 breakpoints seen t(11;14) are dispersed over a 360 kb region between *CCND1* and *myeov*, centromeric to *CCND1* [Janssen et al., 2000]. There is no apparent clustering in the 150 kb major translocation cluster (MTC) as is seen in mantle cell lymphoma [reviewed by Bergsagel and Kuehl, 2001]. As mentioned, the breakpoints do not cluster, but neither are they excluded from the smaller region. Tsai et al. [2008] mapped 10 *CCND1* breakpoints outside of the MTC that were located an average 8.8 bp from CpG dinucleotides. Based on the recent work of Wang et al. [2009], it is feasible that the breaks seen in 11p13 could be attributed not only to aberrant V(D)J recombination or SHM but also to the action of AID on methylated CpGs and late B-cell RAG activity.

BREAKPOINTS MAPPED TO 16q23

Of the five t(14;16) HCMLs characterized, four breakpoints were identified in 16q23. The sequence data collected for these break-

points indicate that all four translocations map within the FRA16D fragile site [Krummel et al., 2000]. Breakpoints identified on der(14) mapped to Sμ, a hybrid switch region, and the J_H region, indicating that off-target CSR, V(D)J recombination, and/or SHM may be responsible for the DSBs in 16q23.

In light of the evidence presented above, it is highly probable that the DSBs generated in putative oncogene partners that are necessary for the translocations evident in myeloma result from off-target activities of AID, RAG, or SHM. However, as well as generating the breaks, it is necessary to repair them, and in the case of translocations, aberrant repair mechanisms may be contributing to the translocation frequency, as DSBs on homologous chromosomes fail to repair with one another.

THE ROLE OF NON-HOMOLOGOUS END JOINING (NHEJ) IN THE GENESIS OF TRANSLOCATIONS IN MYELOMA

Two major repair pathways are responsible for the repair of DSBs in DNA; HR and NHEJ. HR is most active in the late S/G2 phase of the cell cycle and is dependent upon sequence homology of the DSBs. NHEJ can function throughout the cell cycle and requires little or no sequence homology [reviewed by Kotnis et al., 2009]. Similar to most DNA repair pathways, NHEJ involves the following steps: (1) detection of the DSB and stabilization of the DNA ends; (2) resection of damaged DNA; and (3) DNA ligation to repair the phosphodiester backbone. NHEJ is initiated at the sites of DSBs by the heterodimer Ku70/Ku86. The toroidal Ku complex slips onto and binds DNA on either side of the DSB in a sequence-independent manner [Walker et al., 2001]. It appears to "anchor" the ends of the DSBs [Soutoglou et al., 2007] and prevents the use of homologies during recombination [reviewed by Stavnezer et al., 2008]. The Ku complex recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and it has been shown that Ku86 is necessary for the recruitment [Uematsu et al., 2007]. Ku86 possesses a flexible C-terminal "arm," which allows for interactions with DNA-PKcs on both sides of the DSB, promoting trans-autophosphorylation of the kinase. The autophosphorylation of DNA-PKcs modulates its dynamics and stability at DSBs [Hammel et al., 2010], and the Ku:DNA-PKcs complex is believed to "tether" the ends of the DSB. DNA-PKcs recruits the nuclease Artemis, which can act as an exonuclease or an endonuclease [reviewed by Mahaney et al., 2009]. Ku recruits the ligase, XLF:XRCC4:DNA ligase IV to ligate the DNA ends post-processing. Ku can recruit these factors in any order to work on either end of the DSB. Additionally, the nuclease and ligase activities can work on the "top" strand of the break independently of the "bottom" strand [Lieber et al., 2008]. When the classical NHEJ pathway is impaired, an alternative end-joining pathway is operative that appears to utilize microhomology [reviewed by Kotnis et al., 2009].

As previously mentioned, NHEJ is required for the resolution of DSBs generated by CSR and V(D)J recombination. Several factors of the NHEJ pathway are essential for V(D)J recombination, including the Ku heterodimer, DNA-PKcs, and XRCC4, as mice lacking any of these proteins fail to develop B cells [reviewed by Stavnezer et al., 2008]. Although all three proteins are involved in CSR, only Ku is essential for CSR to occur [Casellas et al., 1998; reviewed by Stavnezer et al., 2008]. This suggests the possible use of an alternative repair mechanism to resolve the DSBs caused by CSR.

Ku86v

Of great interest, a study of 14 myeloma patients by Tai et al. demonstrated that 100% expressed a 69 kDa variant of Ku86 (Ku86v) with a truncated C terminus. Two of the patients expressed full-length Ku86 in addition to the variant. All cells retained the ability to bind to DNA ends, although this binding appeared decreased as compared to cells from normal BM samples. The cells expressing Ku86 and Ku86v exhibited normal Ku–DNA-PKcs complex formation but decreased DNA-PKcs kinase activity. Intriguingly, both activities were absent from the cells expressing only Ku86v. This is consistent with the findings that the DNA binding motifs of Ku86 are located in the N-terminus [Osipovich et al., 1999], and the DNA-PKcs binding domain in the C-terminus [Singleton et al., 1999]. The cells expressing only Ku86v displayed increased sensitivity to irradiation and chemotherapeutic agents [Tai et al., 2000]. These results were later challenged by Kato et al. who failed to identify Ku86v in 16 MM cell lines or cells isolated from six patients. They theorized that the Ku86v in the former study was the result of protein degradation during sample preparation; however, no evaluation of Ku86 function was provided [Kato et al., 2002]. Supporting the results of Tai et al. [2000], a recent study identified two HMCLs that consistently express full-length Ku86 as well as Ku86v and suggests that the generation of the variant Ku in MM cells is an innate process. The study implicates serine proteases as important for the generation of Ku86v in intact myeloma cells [Gullo et al., 2008].

Studies in cells isolated from patients with chronic lymphocytic leukemia (CLL) mimic the results of the original myeloma study. A Ku86 doublet consisting of 69 and 71 kDa proteins was detected in B-cell extracts from 4/9 CLL patients. Three of the four patients had low DNA-PKcs activity and sensitivity to a chemotherapeutic agent [Muller and Salles, 1997]. In a separate study of cells isolated from 96 CLL patients, 33% displayed translocations when B cells were stimulated with CD40L. The cells were able to generate many different translocations, but with several recurring break-points in chromosome regions known to harbor oncogenes, including 6p21, 14q32, and 18q21 [Mayr et al., 2006]. The promiscuity of recurrent partners in the translocations of CLL patients is similar to that seen in MM. This suggests that expression of a variant Ku86 protein may be a critical factor in the development of chromosomal instability.

DISCUSSION

Non-random and relative positioning of CTs and genes has implications for the formation of chromosomal translocations, as the free ends of the generated DSBs must be in close contact to rejoin and form a reciprocal translocation [Elliott and Jasin, 2002]. Most importantly, proximity of gene loci in the interphase nucleus affects

the determination of translocation partners [reviewed by Sachs et al., 1997; Meaburn and Misteli, 2007]. This is confirmed in t(9;22) and t(15;17) in human leukemias [Lukasova et al., 1997; Neves et al., 1999], as well as in translocation-prone gene loci in human lymphomas [Roix et al., 2003]. It is highly probable that the oncogene partners involved in the translocations seen in myeloma are located proximally to the *IGH* locus within the cell nucleus. If correct, this could explain the apparent preference for one oncogene over another. For example, the oncogene partner *CCND1*, which translocates with *IGH* at a clinical frequency of 20%, may be more proximal to *IGH* than *c-MAF*, which translocates at a frequency of 5%. Alternatively, considering that gene loci are not static, *CCND1* may be proximal to *IGH* more frequently than *c-MAF*.

It is apparent that the DSBs generated in oncogene partners result from off-target activities of AID, RAG, or SHM (Fig. 1); however, the timing of these events is unclear. Han et al. [2007] have shown that AID is expressed in pre-B and immature B cells of normal wild-type mice, resulting in active SHM and CSR. More recently, Wang et al. [2009] have demonstrated that a subset of activated peripheral B cells with defective NHEJ simultaneously harbor DSBs associated with V(D)J recombination and CSR [Wang et al., 2009]. The involvement of hybrid switch regions in the three most frequent *IGH* translocations in MM suggests that translocations may occur in B cells that have already undergone legitimate CSR, or that undergo CSR at a later stage or outside of the GC. Sequencing studies done in PCs by Taylor et al. [2008] demonstrate ongoing mutation in the *IGH* locus in the area between J_H and Sμ. This suggests that SHM is occurring outside of the GC as a continuing process in end-stage B cells. Collectively, these studies provide evidence to refute the long-held dogma that V(D)J recombination, SHM, and CSR are restricted to specific stages of B-cell development within specific areas of immune tissue. The identification of breakpoints other than those generated by CSR in t(11;14) suggests that recombination mechanisms other than CSR are being utilized. The mechanisms underlying the selective bias of this translocation in IgM myeloma are unknown but may reflect a selective process in which only IgM B cells with t(11;14) are ultimately able to undergo transformation to an IgM myeloma. The stage of B-cell development at which the translocation occurs may influence the type of malignancy that results. It is seemingly apparent that the translocation breakpoints seen in the *IGH* locus are generated at different time points in the B lineage pathway and involve different mechanisms. This may contribute to the promiscuity of translocation partners, because physical proximity to *IGH* may be temporal.

Finally, aberrant repair mechanisms may be contributing to the promiscuity of translocation partners seen in MM. Fifty percent of myeloma cases have loss of *hMLH1*, a protein necessary for the MMR repair [Martin et al., 2006], one of the pathways involved in resolution of DSBs generated by SHM. Two myeloma cell lines demonstrate an impairment of the capacity to repair DSBs via the NHEJ pathway in response to radiation, providing evidence of impaired NHEJ in HMCLs [Yang et al., 2009]. Interestingly, one of these cell lines express Ku86v, in addition to full length Ku 86.

For successful repair to occur, the DNA at the site of DSBs on chromosomes must be held "stable" while the NHEJ machinery repairs the break. Soutoglou et al. have demonstrated that Ku86 is necessary for the positional stability of single DSBs, and that in the absence of Ku, there is an increased frequency of cells displaying clearly separated broken ends (>500 nm) at artificially generated DSBs. The broken ends of DNA in the Ku86 knockdown cells display increased ability to locally diffuse, with the cells showing an increased occurrence of translocations [Soutoglou et al., 2007]. The latter point indicates that DSB repair (albeit aberrant in the case of translocations) is still able to proceed in the absence of Ku, perhaps via the alternative end-joining pathway.

Although the expression of the Ku variant in myeloma is controversial, it is tempting to speculate on the potential role of Ku86v in the promiscuity of translocation partners seen in myeloma. It is not known whether the expression of Ku86v affects positional stability of DSBs. The variant is capable of binding to DNA ends but does not appear to be able to bind to DNA-PKcs. The kinase activity of DNA-PKcs is reduced in patient cells expressing both Ku86 and the variant and is absent in cells expressing only Ku86v [Tai et al., 2000]. It has been shown that Ku86 is essential for the recruitment of DNA-PKcs at DSBs [Uematsu et al., 2007], and it would appear that Ku86v is not able to recruit the kinase in the absence of the C-terminal arm. If the ability of DNA-PKcs to interact in *trans* does indeed contribute to positional stability of DSBs, stability of the DSB ends could be compromised in the presence of the Ku86 variant. This would increase the probability that the chromosome could diffuse to the site of another DSB in a proximal heterologous chromosome. Ku86v may retain its ability to recruit the other members of the NHEJ, allowing NHEJ to proceed, albeit with a non-homologous chromosome. Or, in the absence of DNA-PKcs and its kinase activity, the alternative end-joining pathway could be activated. Alternatively, the expression of both Ku86 and Ku86v may result in a simple competition between the two proteins for binding at DSBs, resulting in a decreased capacity for full-length Ku86 binding. This in turn could result in decreased DNA-PKcs recruitment, which could impair the NHEJ process.

In summary, we speculate that in the evolution of the MM disease process: (1) *IGH* diversification processes [V(D)J recombination, SHM, and CSR] may not be restricted to specific stages of B-cell development or within specific immune tissues, allowing different temporal "windows" in which translocations may occur; (2) off-target activities of the enzymes involved in *IGH* modification processes (AID, RAG, or SHM) contribute to the generation of DSBs on translocation partner loci; (3) spatial proximity of *IGH* and potential translocation partners contributes not only to the selection of a partner but also to the clinical frequency at which a specific translocation occurs; (4) spatial proximity of *IGH* and specific translocation partners may be temporal and contribute not only to partner selection but also to promiscuity of partners; and (5) aberrant repair mechanisms (including the possibility of a Ku86 variant which allows for positional instability at DSBs) may also contribute to the promiscuity of chromosome translocation partners. Studies targeted at mapping spatial and temporal nuclear positioning of *IGH* and its potential translocation partners, identifying temporal expression of *IGH* modification enzymes, and establishing the functionality of a Ku86 variant in translocation events will be necessary to substantiate the above ideas.

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