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Mitotic errors, aneuploidy and micronuclei in Hodgkin lymphoma pathogenesis

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The Reed-Sternberg (RS) cell is the driving force behind Hodgkin lymphoma (HL), a unique malignancy in which the rare RS cell creates an inflammatory microenvironment that recruits a reactive tumor infiltrate. Well-known oncogenic factors such as nuclear factor kappa B (NFκB) signaling and Epstein-Barr virus infection are linked to HL pathogenesis but do not adequately explain the RS cell's key pathologic features of multi-nucleation, abnormalities of centrosome function and number and aneuploidy. Chromosomal instability is also considered a key pathway in the origin of the RS cell, though the molecular mechanisms have largely been a "black box." We demonstrated that the midbody kelch domain protein KLHDC8B protects against mitotic errors, centrosomal amplification and chromosomal instability. Here we discuss how the new findings linking KLHDC8B to mitotic integrity and faithful chromosomal segregation are providing mechanistic explanations for the origin of the RS cell and the molecular pathogenesis of chromosomal instability in HL.

Keywords: Hodgkin lymphoma, Reed-Sternberg cell, aneuploidy, micronuclei, chromothripsis, kelch proteins, mitosis

Abbreviations: GFP, green fluorescent protein; HL, Hodgkin lymphoma; KLHDC8B, kelch-like domain-containing protein 8B; RS, Reed-Sternberg

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The RS Cell Gives HL Unique Properties

Hodgkin lymphoma (HL) is unique among malignancies in that the cancerous cell is rare (as few as one in 100 cells) and instigates the creation of a tumor mass by attracting a stroma of reactive non-clonal lymphocytes, eosinophils and fibroblasts. The pathologic hallmark and malignant cell of classical HL is the multinucleated, giant Reed-Sternberg (RS) cell. RS cells are derived from B lymphocytes of germinal center origin and arise due to a disturbance in cytokinesis by mononuclear Hodkgin cells.[1,2]

Two major signaling pathways are strongly implicated in helping RS cells evade apoptosis. Regulatory and signal transducing proteins in the nuclear factor kappa B (NFκB) pathway are often mutated, resulting in constitutive activation of NFκB.[1,2] Altered signaling through Jak/Stat proteins, in particular gain-of-function in *JAK2*, is strongly implicated as well in the pathogenesis of the RS cell.[1,3] Epstein-Barr virus (EBV) plays a significant role and is implicated in 40 percent of HL cases. EBV helps RS cells avoid apoptosis and recapitulates functions provided by pathogenic signaling mutations.[1,2]

The role of the RS cell as a recruiter of non-clonal cells to form a tumor mass is unique. Beyond avoidance of apoptosis, NFκB and EBV contribute to HL pathogenesis by inducing RS cells to elaborate cytokines and chemokines such as IL-8, IL-12, CCR4 and TGF-β that drive the aggregation of the surrounding inflammatory infiltrate.[4] RS cells' activation of NFκB and tumor necrosis factor receptor family members leads to expression of Th2 cytokines that recruit eosinophils, Th2 cells and fibroblasts.[5] Thus, ability to establish an inflammatory microenvironment is key to HL pathogenesis.

Chromosomal Instability in HL

A key feature of HL, strongly linked to the multinucleated nature of the RS cell,

is the presence of chromosomal aberrations. Case series show strong evidence of chromosomal instability and aberrations in most cases of HL;[6,7] tetraploidy or near-tetraploidy are frequently observed.[8,9] It has been speculated that RS cells or their immediate precursors are derived from a karyotypically aberrant lineage.[1] Although chromosomal instability is strongly implicated in RS cell formation and HL pathogenesis, the mechanisms behind chromosomal instability in HL and the evolution of the RS cell are incompletely known.

KLHDC8B is a mitotically-expressed kelch-domain protein whose deficiency has recently been linked to HL pathogenesis, due to a familial HL pedigree in which a chromosomal translocation disrupted *KLHDC8B*. KLHDC8B localizes to the midbody and has been implicated in multinucleation.[10] The pedigree and the disruption of *KLHDC8B* provided a rare opportunity for insight into the origins of the RS cell. We have performed detailed investigation of the role of KLHDC8B in maintaining mitotic integrity and faithful segregation of chromosomes. We recently reported in the "Journal of Biological Chemistry" that interfering with the function of KLHDC8B leads to significant increases in multinucleation, mitotic errors, abscission failure, centrosomal amplification and aneuploidy.[11] Our ability to recapitulate key features of the RS cell demonstrated the essential roles of mitotic integrity and faithful chromosomal segregation in protecting against HL and shed key light on the molecular mechanisms behind aneuploidy, an essential but poorly-explained component of HL pathology and pathogenesis.

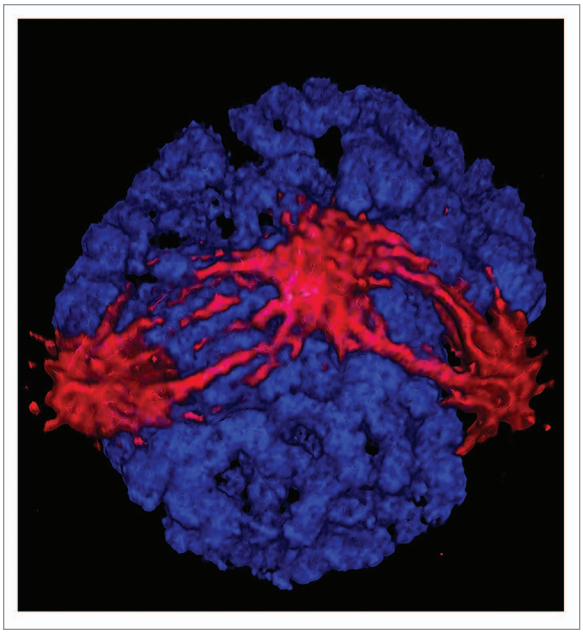

Figure 1. A tripolar metaphase is seen in a mitotic HeLa cell stably expressing the KLHDC8B-GFP fusion protein. Tripolar mitoses, which lead to chromosomal missegregation, are one manifestation of interfering with KLHDC8B's normal function. Chromosomes are stained with DAPI (blue), and spindles are stained with antibodies against α-tubulin (red). Immunofluorescence was performed as described previously.[11] A Z-stack of images was obtained with a Nikon A1R confocal laser scanning microscope, using NIS Elements acquisition software (Nikon). Deconvolution was performed with AutoQuantX (Media Cybernetics). Three-dimensional reconstruction was performed with NIS Elements software.

Extra Centrosomes and Aneuploidy

We used two methods to disrupt KLHDC8B function. We achieved stable knockdown of KLHDC8B by RNA interference in HeLa cells, B lymphoblasts and fibroblasts. In HeLa cells, we also stably expressed a KLHDC8B-GFP fusion protein, which functioned in a dominant-negative role. We observed significant increases in multinucleation, aberrant mitoses, delayed or failed abscission, centrosomal amplification, production of micronuclei and aneuploidy (**Table 1**). Disruption of KLHDC8B also led to asymmetric segregation of daughter nuclei, formation of anucleated daughter cells and multipolar mitotic figures. Thus, we recapitulated the major pathologic features of the RS cell and showed that KLHDC8B helps maintain chromosomal stability.

To the best of our knowledge, our study was the first to report the association of a particular gene with centrosomal amplification and aneuploidy in HL. Centrosomal amplification has been described in a variety of solid tumor and hematologic malignancies.[12] Supernumerary centrosomes are known to cause multipolar mitoses and chromosome missegregation,[13-15] suggesting a direct link between centrosomal amplification and chromosomal instability. We observed increased numbers of multipolar mitoses and metaphases by live cell imaging and fluorescence immunohistochemistry in cells expressing the KLHDC8B-GFP fusion protein, though inadequate numbers were available for statistical analyses. Supernumerary centrosomes, which can yield multipolar mitoses at metaphase (**Fig. 1**), may directly lead to missegregation of chromosomes by forming extra microtubule attachments that interfere with bidirectional mitotic segregation.

Our data suggest a mechanism for chromosomal instability in HL, by which defects in midbody function lead to centrosomal amplification. Centrosomal amplification may be a direct effect of midbody protein dysfunction or may arise due to the accumulation of centrosomes from failed mitoses. Supernumerary centrosomes cause missegregation of chromosomes, in some cases due to multipolar mitoses, leading to cells that have lost or gained chromosomes; the missegregated chromosomes may contain tumor suppressor genes or genes that regulate inflammation, favoring the inflammatory functional profile of the RS cell.

Telomere loss represents an additional potential mechanism for chromosomal instability in the RS cell. Telomere shortening and loss in HL cell lines L-428 and

Table 1. Outcomes of interfering with KLHDC8B function

Characteristic	Method(s) of disruption	Effect	Cell line(s) studied
Multinucleation	Knockdown and fusion protein	2- to 10-fold increase	HeLa, lymphoblast and fibroblast
Centrosomal amplification	Knockdown and fusion protein	4- to 6-fold increase	HeLa and Fibroblast
Aberrant mitotic outcome	Fusion protein	2.4-fold increase	HeLa
Cytokinesis duration	Fusion protein	3.6-fold increase	HeLa
Aneuploidy	Knockdown	1.5- to 2.5-fold increase	Lymphoblast and Fibroblast
Micronucleation	Fusion protein	2.5-fold increase	HeLa

L-1236[16] may lead to breakage-bridge fusion (BBF) cycles, in which sister chromatids without telomeres fuse to each other and subsequently form internuclear DNA bridges during anaphase.[17,18] The bridges are subsequently broken by forces of tension, leading to uncapped chromatids that are free to repeat the cycle again, resulting in translocations and aneuploid nuclei. BBF cycles may account for unequal and disrupted distribution of chromosomes between nuclei, resulting in RS cell nuclei that may be "chromosome-poor," or aneuploid.[19] One caveat, though, is that L-428 and L-1236 have markedly aberrant karyotypes. They may be less informative about the initial steps leading to chromosomal instability than they are indicative of the damage that can be inflicted by chromosomal instability processes in RS cells.

Micronuclei, Pulverized Chromosomes and DNA Damage

Interfering with KLHDC8B's function resulted in an increased incidence of micronuclei, which originate from chromosome fragments that arise due to DNA damage or missegregated chromosomes.[20] The whole or partial chromosomes are sequestered into structures similar to but much smaller than normal nuclei (**Fig. 2**). Given that we observed increased rates of aneuploidy, it is more likely that the micronuclei observed in our study are composed of whole chromosomes. Micronucleus formation has previously been associated with centrosomal amplification and spindle assembly defects.

Aneuploidy and micronuclei are established correlates of increased cancer risk.[21] The recent discovery of chromothripsis (*thripsis* means "pulverization" or "shattering into pieces" in Greek), the phenomenon of a single chromosome pieced together from tens to hundreds of rearranged fragments, gives insight into how chromosomal instability can trigger carcinogenesis over a brief mutational time span.[22] BBF cycles and impaired double-strand break repair may contribute to chromothripsis, but the most promising model for the generation of chromothriptic chromosomes is based on micronuclei. It is hypothesized that the DNA of chromosomes or chromosome fragments in micronuclei does not appropriately condense at the G2/M cell cycle checkpoint, and the chromosomes are then pulverized during mitosis. The DNA is subsequently reassembled into patchwork chromosomes that are ultimately reincorporated into the main cellular nucleus.[23] Additionally, micronuclei suffer from inadequate acquisition of DNA repair and synthesis machinery, further contributing to double-strand breaks, pulverization and thus the rapid accumulation of mutations.[24,25] KLHDC8B protects against micronucleus formation and thus possibly chromothripsis, which appears to be a promising candidate mechanism by which the aneuploid RS cell acquires the multiple mutations necessary to allow evasion of apoptosis and establishment of an inflammatory microenvironment.

The concept of a micronuclear environment that is deficient in DNA damage repair and DNA synthesis mechanisms suggests a link between the functions of faithful chromosomal segregation and

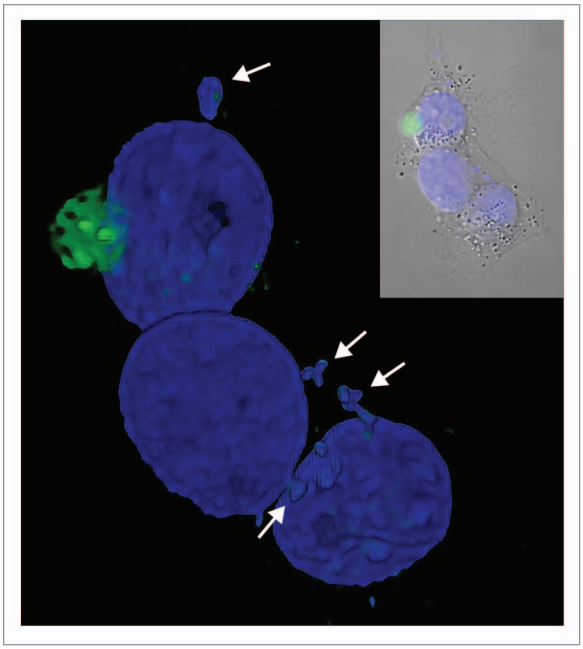

Figure 2. Several micronuclei (arrows) are observed in a multinuclear HeLa cell expressing the KLHDC8B-GFP fusion protein. An aggregate of the fusion protein is seen (green). Chromosomes are stained with DAPI (blue). The inset demonstrates the DIC image of the cell, including the green and blue channels. Immunofluorescence was performed as described previously.[11] A Z-stack of images was obtained with a Nikon A1R confocal laser scanning microscope, using NIS Elements acquisition software (Nikon). Deconvolution was performed with with AutoQuantX (Media Cybernetics). Three-dimensional reconstruction was performed with NIS Elements software.

DNA damage repair. Similar connections exist within the identities of the many proteins performing these two seemingly separate tumor suppressor functions. BRCA1 and BRCA2 are well-known for their roles in signaling DNA damage and directly participating in DNA repair, respectively, but both proteins also localize to the midbody and protect against aneuploidy.[26,27] The nucleic-acid binding proteins of the NF90/NF45 complex help repair double-strand breaks by non-homologous end joining, yet they also protect against cytokinesis failure and multinucleation.[28] These findings raise the intriguing possibility that KLHDC8B plays a tumor suppressor role extending beyond guarding against mitotic errors and aneuploidy, perhaps in DNA damage repair. We plan to investigate the possible roles of this midbody protein, a relative newcomer to the field of cancer biology, with subsequent cellular and animal models of KLHDC8B dysfunction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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