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Note

Manipulating Mitotic Recombination in the Zebrafish Embryo Through RecQ Helicases

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ABSTRACT

RecQ DNA helicases resolve Rad-51-mediated recombination and suppress aberrant homologous recombination. RecQ gene loss is associated with cancer susceptibility and increased mitotic recombination. We have developed an *in vivo* assay based on a zebrafish pigment mutant for suppression of RecQ activity, and demonstrate that zebrafish RecQ genes have conserved function in suppressing mitotic recombination.

HELICASES function in DNA replication, repair, recombination, and RNA transcription (van Brabant *et al.* 2000); helicase-catalyzed strand separation is usually coupled to adenosine triphosphate (ATP) hydrolysis. RecQ-related helicases contain a characteristic RecQ C-terminal (RQC) domain, which likely interacts with other proteins (Hickson 2003; Khakhar *et al.* 2003), and the RNAseD C-terminal (HRDC) domain, thought to be involved in binding the nucleic acid substrate (Liu *et al.* 1999). Among other functions, RecQ helicases suppress homologous recombination at stalled replication forks (Khakhar *et al.* 2003). In response to lesions that impede replication, RecQ-deficient cells use homologous recombination to restart replication, leading to accumulation of recombination-dependent cruciform structures at damaged forks (Liberi *et al.* 2005).

Mutations in human RecQ-related genes, *BLM*, *WRN*, and *RECQL4*, give rise to Bloom syndrome, Werner syndrome, and Rothmund–Thompson syndrome, respectively (Ellis *et al.* 1995; Kitao *et al.* 1999; Yu *et al.* 1996). Patients share susceptibility to cancer, implicating RecQ genes in tumor suppression. The cellular hallmark of Bloom syndrome is increased mitotic recombination, evidenced by sister chromatid exchange (SCE) and recombination between homologs. Loss of BLM also correlates with an increased rate of targeted homologous recombination (Luo *et al.* 2000; Wang *et al.* 2000). Loss of another RecQ family member, Recql5, further increases the rate of SCE in *Blm*$^{-/-}$ cells (Hu *et al.* 2005; Wang *et al.* 2003).

We identified ESTs encoding two zebrafish RecQ-related genes, most closely related to *Blm* and *Recql5* genes from other species (data not shown). *blm* and *recql5* were expressed maternally and ubiquitously through somitogenesis. Thereafter, they displayed complex, largely overlapping patterns of tissue-specific transcription, prominent in the heart, brain ventricles, otic vesicle, retina, and somites through 5 days post fertilization (dpf) (data not shown).

Mitotic recombination can lead to distal loss of heterozygosity and cells homozygous for a mutation initially present on one homolog. Mutants for the zebrafish pigment gene *golden* (*gol*) (Lamason *et al.* 2005) have reduced melanin accumulation, apparent in the retinal pigment epithelium (RPE) at 3 dpf. The induction of *gol*$^{-}$ cells in *gol*$^{+/-}$ heterozygotes has been used to estimate the size of the RPE precursor pool and the timing of their commitment (Streisinger *et al.* 1989) and as the basis of a genetic screen for mutants with genomic instability (Moore *et al.* 2004; Moore *et al.* 2006). We have used embryos heterozygous for *gol*^{b1}, a null allele maintained on the AB genetic background (Johnson and Zon 1999), in an assay for the suppression of Blm and Recql5 function through injection of putative dominant negative RNAs.

Several BLM mutations reportedly have dominant negative activity, on the basis of their ability to cause mislocalization of wild-type (WT) BLM (Wang *et al.*

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A                    *
DR BLM     TFVLMPTGGGKSLCYQLPACVSAGVTVVISP   666
MM BLM     CFILMPTGGGKSLCYQLPACVSPGVTIVISP   723
HS BLM     CFILMPTGGGKSLCYQLPACVSPGVTVVISP   715

B                    *
DR BLM     GEHTFNTSFCKEHPEVICDNCARPNKYKSRN  1087
DR BLM     GEKGFNPDFCKKYPDVSCDNCCKTKDYKTKD  1084
DR BLM     GENGFNPDFCKKHPDVSCDNCCKTKDYKTRD  1076

C                          *
DR RECQL5  LQGDRDVFVCMPTGAGKSLCYQLPALMASGV    60
MM RECQL5  VKGAEDVFVCMPTGAGKSLCYQLPALLASGI    72
HS RECQL5  VKGNKDVFVCMPTGAGKSLCYQLPALLAKGI    72
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Figure 1.—Portions of Blm and Recql5 protein sequences for zebrafish (DR), mouse (MM), and human (HS), indicating positions of conserved residues altered in mutant RNAs for injections; identical residues are shaded. The conserved lysine residues in the ATP-binding sites, mutated in Blm^{K706T} (A) and $Recql5^{K46T}$ (C), and the conserved cysteine residue in the RQC domain, mutated in Blm^{C1066S} (B), are indicated by asterisks. Two zebrafish ESTs (accession nos. CB352479 and BG883342) were homologous to the 5′- and 3′-ends of human *BLM* cDNA, respectively, with an internal overlap of 1.2 kb. The full-length zebrafish *blm* coding sequence was assembled by PCR. One EST (accession no. BG304090) containing the entire coding sequence of zebrafish *recql5* was identified (data not shown), corresponding to the longest (β) splicing form containing a nuclear localization signal (Shimamoto *et al.* 2000).

2001) or to increase the rate of SCE in WT cells (Neff *et al.* 1999). However, one such mutation (BLM^{C1055S}) was first described as a homozygous mutation in a patient (Ellis *et al.* 1995), suggesting that it does not act dominantly *in vivo*. We generated constructs encoding mutated zebrafish proteins, blm^{C1066S} ($blm^{C>S}$) and blm^{K706T} ($blm^{K>T}$), corresponding to described human mutations, and mutant $recql5^{K46T}$ ($recql5^{K>T}$) was similarly generated in the conserved ATP-binding site of the helicase domain (Figure 1). WT and mutant RNAs were injected into $gol^{+/-}$ embryos at the one-cell stage, and the larvae assayed at 3 dpf for hypopigmented RPE cells (Figure 2). Injection of WT *blm*, $blm^{C>S}$, or WT *recql5* RNAs gave rise to hypopigmented cells in the RPE at similar, low frequences (Table 1), indicating that $Blm^{C>S}$ does not have a specific dominant negative effect. Rather, WT Recql5, WT Blm, and $Blm^{C>S}$ may nonspecifically disrupt helicase function when ectopically expressed at high levels. In contrast, injection of $blm^{K>T}$ or $recql5^{K>T}$ RNAs induced RPE mosaicism at significantly higher frequencies (Table 1). Consistent with the mutant proteins acting as dominant negatives, suppression of *recql5* function through injection of an antisense morpholino also led to RPE mosaicism (Figure 2F; Table 1).

Clones of $gol^{-/-}$ cells in a $gol^{+/-}$ embryo could arise by somatic mutation at the WT allele of *gol*, gene conversion, deletion of the region encompassing the WT *gol* locus, mitotic nondisjunction, or mitotic recombination between homologs proximal to *gol*. Prior studies on

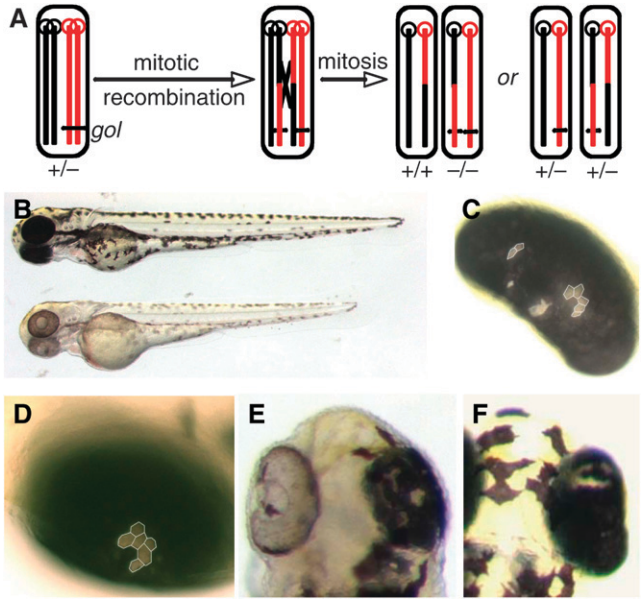

Figure 2.—Suppression of Blm or Recql5 activity induces somatic mosaicism. (A) Diagram of a mitotic recombination event producing $gol^{-/-}$ cells. (B) Note the difference in eye pigmentation between a $gol^{+/-}$ (top) and $gol^{-/-}$ (bottom) larva at 3 dpf. (C–E) Examples of mosaicism resulting from injection of either $blm^{K>T}$ or $recql5^{K>T}$ RNA are shown. Some RPE cells are outlined to indicate the number of $gol^{-/-}$ cells in the clones (C and D). (E) Dorsal view of one injected fish at 3 dpf, with hypopigmented RPE cells in one entire eye and several smaller clones in the other eye. (F) Suppression of Recql5 through antisense morpholino injection can also induce RPE mosaicism, seen in this dorsal view of a larva at 3 dpf. Point mutations were introduced into *blm* and *recql5* by megaprimer PCR (Ke and Madison 1997). RNAs were transcribed *in vitro* and injected as previously described (Xie and Fisher 2005).

BLM point to mitotic recombination as the likely mechanism (Luo *et al.* 2000). To exclude some of these mechanisms, we genotyped $gol^{-/-}$ RPE cells from mosaic embryos. We crossed $gol^{-/-}$ fish on the AB genetic background to WIK fish (Johnson and Zon 1999) to generate $gol^{+/-}$ embryos on a polymorphic background and injected them with $blm^{K>T}$ and $recql5^{K>T}$ RNAs. From larvae with large patches of hypopigmented RPE cells, genomic DNA was prepared separately from dissected RPE cells and the remainder of the embryo. In two independent cases, only AB-specific alleles were detected from the RPE tissue for markers close to *gol*, while a proximal marker gave rise to AB and WIK bands; the remainder of the embryo was heterozygous for all three markers (Figure 3). These results are inconsistent with gene conversion, somatic mutation, or mitotic nondisjunction. They are consistent with mitotic recombination events proximal to *gol*, although we also cannot rule out small deletions encompassing *gol*.

Mitotic recombination proximal to *gol*, in any cell that eventually gives rise to RPE, could give rise to a clone of gol^{-} cells that would be scored in our assay. At 3 dpf, the RPE consists of a single layer of ~550 polygonal cells in

TABLE 1

Suppression of Blm or Recql5 activity induces formation of *gol*⁻ cells in *gol*$^{+/-}$ embryos

RNA injected (pg)	% RPE clones (no. of embryos)	% abnormal
blm (100)	0.0 (187)	11.0
blm (500)	0.1 (710)	16.0
blm$^{C>S}$ (100)	0.0 (198)	9.1
blm$^{C>S}$ (500)	0.5 (619)	14.0
recql5 (100)	0.0 (179)	6.1
recql5 (500)	0.6 (326)	15.0
blm$^{K>T}$ (60)	1.7* (178)	3.4
blm$^{K>T}$ (120)	1.9* (261)	7.3
blm$^{K>T}$ (200)	3.4* (146)	12.0
recql5$^{K>T}$ (60)	2.1* (327)	5.8
recql5$^{K>T}$ (120)	2.4* (252)	6.3
blm$^{K>T}$+*recql5*$^{K>T}$ (15 each)	2.3* (219)	4.1
blm$^{K>T}$+*recql5*$^{K>T}$ (30 each)	3.5* (171)	4.1
blm$^{K>T}$+*recql5*$^{K>T}$ (60 each)	3.6* (611)	7.4
blm$^{K>T}$+*recql5*$^{K>T}$ (100 each)	3.0* (267)	21.0
recql5-MO (1 ng)	1.6* (501)	N/A

The indicated RNAs were injected into *gol*$^{+/-}$ embryos at the one-cell stage; at 3 dpf, embryos were scored for the presence of clones of hypopigmented cells in the RPE (% RPE clones). The number of embryos that developed with gross morphological abnormalities, such as shortened axis, reduced head size, or general developmental delay, were also scored (% abnormal). A standard one-sided test for equality of probabilities in binomial data indicated that all injections with *blm*$^{K>T}$ and *recql5*$^{K>T}$ RNAs or with the *recql5* morpholino differed significantly from injection of WT RNAs. *$P < 0.001$.

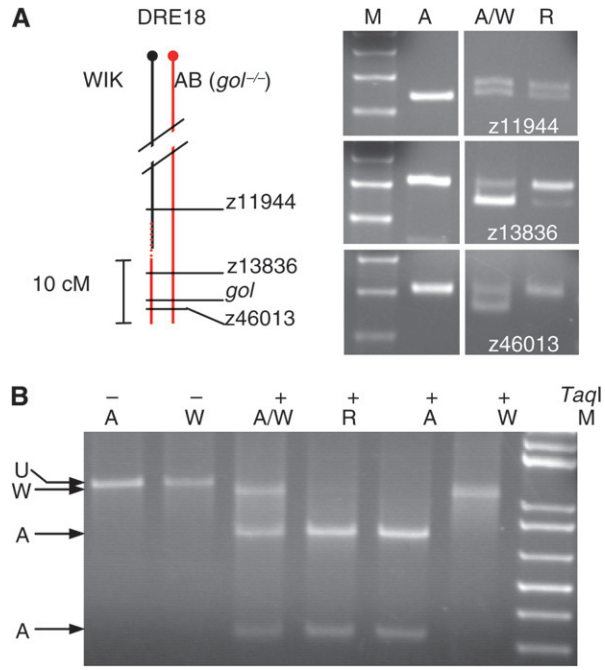

Figure 3.—Molecular evidence for mitotic recombination in mosaic *gol*$^{+/-}$ embryos. *gol*$^{-/-}$ fish were crossed with WIK fish, and 30 pg each of *blm*$^{K>T}$ and *recql5*$^{K>T}$ RNAs was injected into the resulting embryos. Larvae were fixed, large patches of hypopigmented RPE cells dissected out, and DNA prepared from RPE cells and remainder of the larva. (A) For one mosaic larva, DNA samples from the parental AB strain (indicated as A above gel lanes), the bulk of the larva (A/W), and the dissected RPE cells (R) were typed for three SSLP markers on chromosome 18; their positions relative to *gol* are indicated in the map (left). For the proximal marker (z11944), both the RPE cells and the rest of the larva are heterozygous for the AB and WIK parental alleles. For distal markers (z13836 and z46013), RPE cells show predominantly the AB allele, while the rest of the embryo is heterozygous. This pattern is consistent with a recombination between z11944 and z13836, as indicated by the homozygous red chromosomal segment in the diagram. (B) DNA samples from a second mosaic larva were typed for a SNP in intron 5 of the *gol* gene, revealed by *Taq*I restriction digest. DNA from the entire embryo is heterozygous for the SNP, while the RPE cells display only the SNP associated with the *gol*^{b1} allele. M, marker lane.

each eye, which arise from ~40 specified precursors (Streisinger *et al.* 1989). Mitotic recombination in one precursor cell would generate a clone of ~14 *gol*$^{-/-}$ cells ($1100/40 \times 0.5$), consistent with the clone size in many mosaic embryos. However, in cases where *gol*$^{-/-}$ cells composed one-fourth to one-half of total RPE cells (Figure 2E), recombination may have occurred as early as the one-cell stage. In addition, the smallest clone in our assay contained only two cells (Figure 2D), suggesting that recombination events continue to occur for some time after the RPE precursors are specified. Thus, it seems likely that suppression of Blm and Recql5 activities can result in mitotic recombination during any of the early cell cycles.

Genetic mosaics allow for the study of pleiotropic gene function and analysis of cell lineage and cell autonomy. These are created in other experimental organisms through site-specific Flp recombinase (Golic and Lindquist 1989; Xu and Rubin 1993) or Cre–loxP-mediated recombination (Gu *et al.* 1994; Zong *et al.* 2005). We show here that suppression of RecQ helicases can also generate somatic clones of homozygous mutant cells. Through Blm and Recql5 suppression in germ cells, a similar approach might also be used to generate zebrafish embryos entirely lacking a maternally required gene product from a heterozygous female, with a method less cumbersome than that currently employed (Ciruna *et al.* 2002). To this end, we fused mutant *blm* or *recql5* coding sequences to the 3′ UTR of *nanos-1* (*nos1*) (Koprunner *et al.* 2001). Chimeric RNAs were injected, together with *gfp–nos1*–3′ UTR RNA, to enable visualization of germ cells. At the lowest RNA levels, GFP$^+$ germ cells appeared normal in the large majority of embryos (Table 2). Progressively fewer embryos displayed normal germ cells at higher RNA doses, but >80% of embryos were still grossly normal, showing that deleterious effects were largely confined to the germ cells. Injected larvae showing normal numbers of germ cells were raised to adults; these were fertile and their offspring developed normally (data not shown), indicating that germ cell function was not compromised.

TABLE 2

Suppression of Blm and Recql5 activities allows normal germ cell development

RNA injected (pg)	% abnormal (no. of embryos)	Normal	
		% GFP−	% GFP+
GFP (50)	4.8 (84)	2.4	93
blm$^{K>T}$ (80)	7.8 (51)	20	73[a]
recql5$^{K>T}$ (80)	11 (65)	7.7	82[a]
blm$^{K>T}$+*recql5*$^{K>T}$ (40 each)	12 (43)	14	74[a]
blm$^{K>T}$+*recql5*$^{K>T}$ (80 each)	10 (48)	29	60
blm$^{K>T}$+*recql5*$^{K>T}$ (160 each)	17 (63)	67	16

The mutant *blm* or *recql5* cDNA was inserted into pSP64GFP3′UTRnos1, replacing the *gfp* coding sequence and allowing transcription of chimeric RNAs containing the 3′ UTR sequence of *nanos-1* gene (Koprunner *et al.* 2001). The indicated RNAs were injected as previously described; all injections included 50 pg of *gfp-nos1* 3′ UTR RNA to mark the germ cells with GFP. The embryos that developed with gross morphological abnormalities are indicated in the "% abnormal" column; within the grossly normal embryos, "GFP−" indicates those with no apparent germ cells, and "GFP+" indicates embryos with number and location of germ cells similar to control embryos.

[a] Embryos saved for stocks, to assess fertility.

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