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Ubc13 dosage is critical for immunoglobulin gene conversion and gene targeting in vertebrate cells

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ABSTRACT

In contrast to lower eukaryotes, most vertebrate cells are characterized by a moderate efficiency of homologous recombination (HR) and limited feasibility of targeted genetic modifications. As a notable exception, the chicken DT40 B cell line is distinguished by efficient homology-mediated repair of DNA lesions during Ig gene conversion, and also shows exceptionally high gene-targeting efficiencies. The molecular basis of these phenomena is elusive. Here we show that the activity levels of Ubc13, the E2 enzyme responsible for non-canonical K63-linked polyubiquitination, are critical for high efficiency of Ig gene conversion and gene targeting in DT40. Ubc13$^{+/-}$ cells show substantially lower homology-mediated repair, yet do not display changes in somatic hypermutation, overall DNA repair or cell proliferation. Our results suggest that modulation of the activity of K63-linked polyubiquitination may be used to customize HR efficiencies in vertebrate cells.

INTRODUCTION

Maintenance of genetic information is a fundamental requirement for organismic survival. Accordingly, DNA repair mechanisms are highly conserved, including excision repair for removal of altered bases, mismatch and post-replication repair for replication-associated problems, and double-strand break repair for re-ligation of breaks disrupting helix continuity (1). The latter encompasses non-homologous end joining (NHEJ) and homologous recombination (HR), which both restore the helix but differ with respect to fidelity (2). For most forms of DNA damage, several repair mechanisms act in a redundant, cooperative or even competitive manner to ensure optimum accuracy and efficiency of rectification.

Despite this variety of DNA repair mechanisms, some instances call for increased genetic variability. A prototypic example is the vertebrate adaptive immune system that relies on intricate genetic mechanisms to generate a vast array of antigen receptors to fight invading pathogens (3). Initially, V(D)J-recombination generates numerous patchwork sequences encoding antigen receptors with different specificity. For further diversification, B lymphocytes may introduce templated or non-templated changes into these V(D)J joints by immunoglobulin gene conversion (Ig GC) or somatic hypermutation (SHM) (4,5). Both processes are initiated via cytidine deamination by activation-induced deaminase [AID (6)], which precipitates either HR with upstream pseudogenes during Ig GC, or random mutagenesis during SHM. The relative incidence of these two processes differs between species and may be determined by the overall capacity of the cells to perform HR (7).

The DT40 cell line, derived from a chicken bursal B cell, constitutively diversifies its endogenous Ig light chain genes by Ig GC (5). Probably as a side effect, the cells also display very high activity of HR, and the resultant exceptional targeting efficiencies have made DT40 a valuable tool to study vertebrate gene function. The molecular basis for high HR activity in DT40 has remained elusive. Indeed, complete inactivation of HR has proven incompatible with DT40 survival (8). Inactivation of non-essential HR factors impairs DNA damage tolerance of the cells and leads to a shift from Ig GC to SHM (7). A treatment that would selectively affect HR capacity of the cells without detrimental effects on other parameters has not been identified to date.

It is well recognized that HR avoids aberrant repair of some DNA lesions. In case of DNA double-strand breaks, HR is able to correctly restore even challenging lesions

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based on an undamaged homologous copy, while direct NHEJ is often error-free, but may also lead to deletions or chromosomal translocations (2). In case of DNA changes affecting only one strand, such as bulky adducts or abasic sites, homology-based error-free bypass may be employed during replication to avoid mutagenic bypass by translesion synthesis (9). The choice between these options is regulated by the Rad6 pathway: monoubiquitination of proliferating cell nuclear antigen (PCNA) by Rad6/Rad18 recruits translesion polymerases, and K63-linked PCNA polyubiquitination by Ubc13/Mms2 is the basis for error-free bypass via homology-based template switching to the undamaged sister chromatid (10). Ubc13 also plays a critical role in HR, due to its interaction with RNF8/RNF 168 and subsequent ubiquitination events at sites of DNA damage (11,23).

In the present study, we have analysed the impact of partial interference with Ubc13 function in DT40 cells. We show that Ubc13 heterozygosity selectively interferes with Ig GC and gene targeting efficiency, without affecting the other parameters studied. Our findings suggest that quantitative interference with K63-linked ubiquitin conjugation, which plays a key role in HR (8,11), may be used to fine-tune the targeting capacity of vertebrate cells.

MATERIALS AND METHODS

Cells, vectors and targeting

DT40Cre1 and DT40ψV- cells were cultured as described before (12). For construction of a Ubc13 targeting vector, the left and right arms were amplified with 13Ub1, 13Ub2, 13Ub3 and 13Ub4 (for primer sequences, see Table 1), and cloned into pBluescriptKS together with loxP-flanked cassettes for puromycin or blasticidin resistance (13). For reconstitution of Ubc13, its coding sequence was amplified using 13Ub8 and 13Ub9, cloned into the pExpress vector, and the resultant Ubc13 cassette was cloned into the Nhe1 site of ploxGPT (13).

Transfection of DT40 cells and screening for targeted integration with the primers 13Ub101 and Pu4 or Bs1 were done as described (12). For Southern blots, genomic DNA was cut with *Pst*I and fragments were detected with a probe amplified by the primers 13Ub201 and 13Ub202.

Assays for Ig GC and SHM

Analyses of Ig GC and SHM were done as before (12), with the following exceptions: cells were cultured for 6 weeks after subcloning before FACS analysis for Ig GC, and for 2 more weeks for sequencing of the Ig light chain genes. For SHM analysis, cells were cultured for 14 days after subcloning for FACS analysis and stained with anti-chicken-IgM-PE (Southern Biotec, Birmingham, Alabama). SHM sequence analyses were carried out after 4 more weeks of culture.

Table 1. Sequences of oligonucleotides used in this study

Name	Sequence
13Ub1	5′-gggctcgagtccagcctggccttggctgcttcca-3′
13Ub2	5′-gggggatcccaaactgtttaacgtaagcactctg-3′
13Ub3	5′-gggggatccgaataaaagcccagacatcttcagt-3′
13Ub4	5′-gggactagtaccttcagacacagcactactaccg-3′
13Ub8	5′-gaagctagcgccaccatggccgggctgcccc-3′
13Ub9	5′-gggagatctgtcttggcagaacaggagaagtgat-3′
13Ub101	5′-tagaatcatacagtggtttgggttgga-3′
Pu4	5′-cagcgcccgaccgaaaggagcgca-3′
Bs1	5′-cgattgaagaactcattccactcaaatatataccc-3′
13Ub201	5′-gtgtaagaggacaagcaagatac-3′
13Ub202	5′-agttgggctaaatgaccttcg-3′

Analysis of proliferation and cell survival

For analysis of cell proliferation, cells were labelled with the Vybrant™ CFDA SE Cell Tracer Kit (Invitrogen, USA) and FACS analyses were performed every 10–12 h. The sensitivity of the cells to DNA damaging drugs was tested using a modified colony survival assay according to a published protocol (14). Cells were treated with the drugs in six well plates with 1×10^6 cells in 5 ml per well for 2 h at 37°C. The cells were then washed twice and resuspended in 10 ml. One hundred microlitres of this suspension and of a 1:10 and 1:100 dilution were plated in duplicate on wells with 5 ml 1.5% methyl cellulose, followed by 10–12 days of culture at 37°C. For treatment with ionizing radiation, cells were serially diluted, plated on methylcellulose, incubated at 37°C for 2 h and then irradiated (137 Cs γ-ray source, MDS-Nordion, Canada).

RESULTS

We have recently shown that initiation of the Rad6 pathway by Rad18 is important for SHM (12). To assess the role of PCNA polyubiquitination in Ig diversification, we wished to inactivate Ubc13 in DT40, using vectors targeting exons 2–4 (Figure 1A). Inactivation of the first Ubc13 allele in DT40 Cre1 cells could be achieved with high efficiency, but direct inactivation of the second Ubc13 allele proved impossible, as no targeted clones could be identified despite repeated attempts. We noticed, though, that in contrast to the parental Cre1 cells, the Ubc13$^{+/-}$ clones barely converted from the original surface (s)Ig− configuration due to a frameshift in the expressed Ig light chain gene to a sIg+ state that is indicative of ongoing Ig GC (15). We therefore set out to analyse the effects of Ubc13 heterozygosity on Ig diversification in DT40.

To this end, an experimental set comprising two independent heterozygous clones and derivatives containing an Ubc13 over-expression vector was generated. The genetic status of the cells was confirmed by Southern Blot (Figure 1B), and Western Blots revealed that Ubc13 expression levels were decreased in heterozygous clones and restored to or beyond normal levels in the reconstituted clones, while AID levels remained constant (Figure 1C).

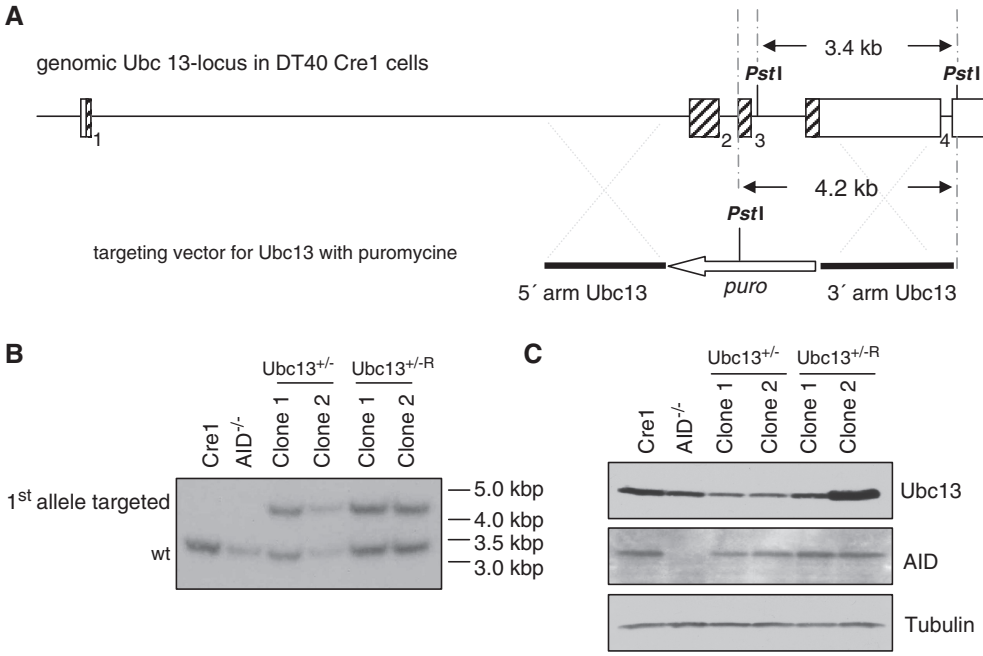

Figure 1. Targeting and reconstitution of Ubc13. (**A**) Targeting strategy for Ubc13 in DT40 cells. Boxes indicate exons, shaded areas correspond to coding sequences. Restriction sites and fragment sizes for southern analyses are shown. Bold lines correspond to homologous arms of the targeting vector. (**B**) Southern Blot analysis of the DT40 clones used for analysis of Ig GC. 2 independent heterozygous ($Ubc13^{+/-}$) and reconstituted clones ($Ubc13^{+/-R}$) were employed. (**C**) Western Blot for Ubc13 and AID expression in the clones analysed in (B).

A quantitative Ig GC assay based on generation of sIg+ cells in multiple independent sIg− subclones (15) indeed showed a significantly decreased Ig GC efficiency in the Ubc13 heterozygous cells that was not evident in the reconstituted derivatives (Figure 2A and B). To confirm that Ubc13 levels are critical for efficient Ig GC in DT40, we polymerase chain reaction (PCR)-amplified, cloned and sequenced Ig light chain genes from representative subclones of the respective genotypes. In the parental DT40 Cre1 cells, Ig GC events could easily be identified in 10 of 24 sequences (Figure 2C). In the heterozygous cells, however, only one Ig GC event was found in one clone, while in the other clone a duplication event and no evidence of Ig GC could be identified (Figure 2C). Thus, even moderate interference with Ubc13 function substantially obstructs the efficiency of Ig GC in DT40.

Interference with HR in DT40 cells may affect the capacity of the cells to proliferate or survive upon DNA damage (8,11). Assays of proliferative capacity of the cells by CFSE labelling revealed no differences, as generation times averaged around 11 h in all cell types tested (Supplementary Figure S1A). This indicates that HR must be functional to an extent allowing the cells to pass critical events in S/G2 that would otherwise cause cell death in DT40 (8). Also, sensitivity of the $Ubc13^{+/-}$ cells to cisplatin, mitomycin C and ionizing radiation was not affected, although $Rad18^{-/-}$ control cells behaved as expected (Supplementary Figure S1B–D). Accordingly, Ubc13 heterozygous cells show normal proliferation and DNA damage tolerance, but are characterized by a substantially diminished Ig GC activity.

An impaired function of factors involved in HR, such as Ubc13, often not only causes a decrease in Ig GC but a concomitant increase in SHM activity (7). The sequence analysis shown in Figure 2C, however, indicated no increased mutagenesis in the variable region. To directly assess the effect of Ubc13 heterozygosity on SHM, we inactivated one Ubc13 allele in DT40ψV$^-$ cells lacking the pseudogenes required for Ig GC, which only diversify their V genes by SHM (16). Curiously, for unknown reasons, only a heterozygous clone was identified among 65 clones screened in different targeting attempts, and reconstitution was performed as above. SHM assays based on subcloning sIg+ cells from each genotype and determining loss of sIg expression due to deleterious mutations after 2 weeks of culture (Figure 3A) revealed no significant difference in the $Ubc13^{+/-}$ clone or the reconstituted derivative, and neither did sequence analyses (Figure 3B). These data imply that Ubc13 heterozygosity impairs homology-mediated repair of AID-induced lesions without shifting repair balance to more substantial mutagenesis.

Competition between homology-directed and TLS-based mutagenic repair mostly occurs when DNA lesions are encountered during replication (9,10). Ubc13 heterozygosity may not limit its activity to an extent perturbing the error-free arm of post-replicative repair, i.e. PCNA polyubiquitination. While excision of AID-induced uracils by UNG leads to abasic sites that can give rise to translesion synthesis or homology-based error-free bypass, they can also be converted to DNA single- and double-strand breaks by APE1 (17). On these lesions, repair by HR may compete with NHEJ, and the

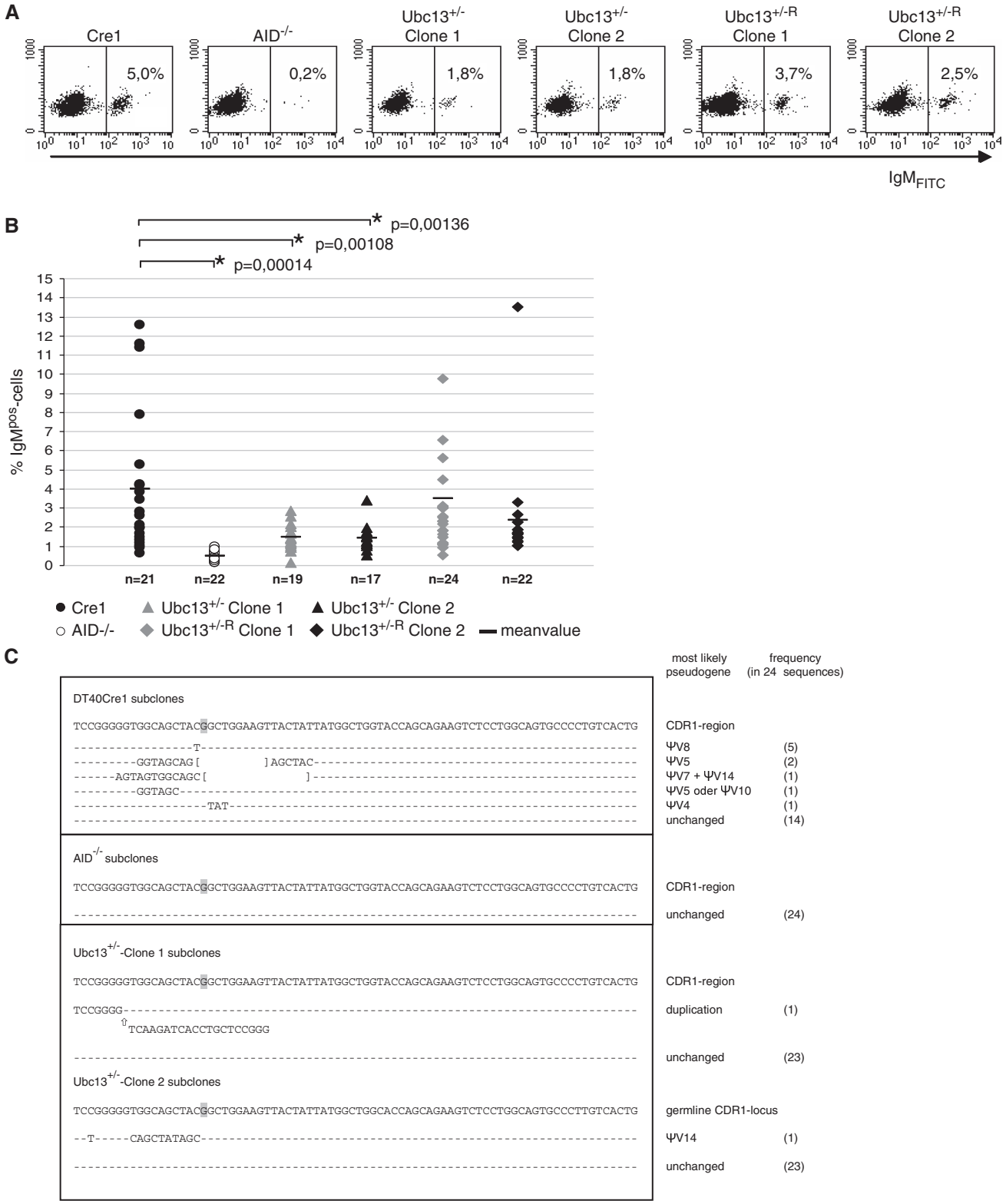

Figure 2. Ubc13 dosage affects Ig GC. (**A**) FACS analysis for sIg expression of representative single cell clones of the cells with the indicated genotypes. Percentage of sIg+ cells are given. (**B**) Percentage of sIg+ cells in individual single-cell clones of the indicated genotypes. Total numbers of clones analysed are given below the genotype. Bars represent mean values. Brackets and stars mark statistically significant differences (Student's *t*-test). *P*-values for Cre1 compared to the reconstituted clones were 0.20367 and 0.07022, respectively. (**C**) Sequence analysis of the rearranged light chain gene in representative clones of the cells analysed in (B). The shaded letter marks the original frameshift mutation in DT40Cre1 cells. Brackets mark deletions. The frequency of the events shown among a total of 24 sequences each, as well as the most likely pseudogene with which recombination occurred, are indicated on the right side.

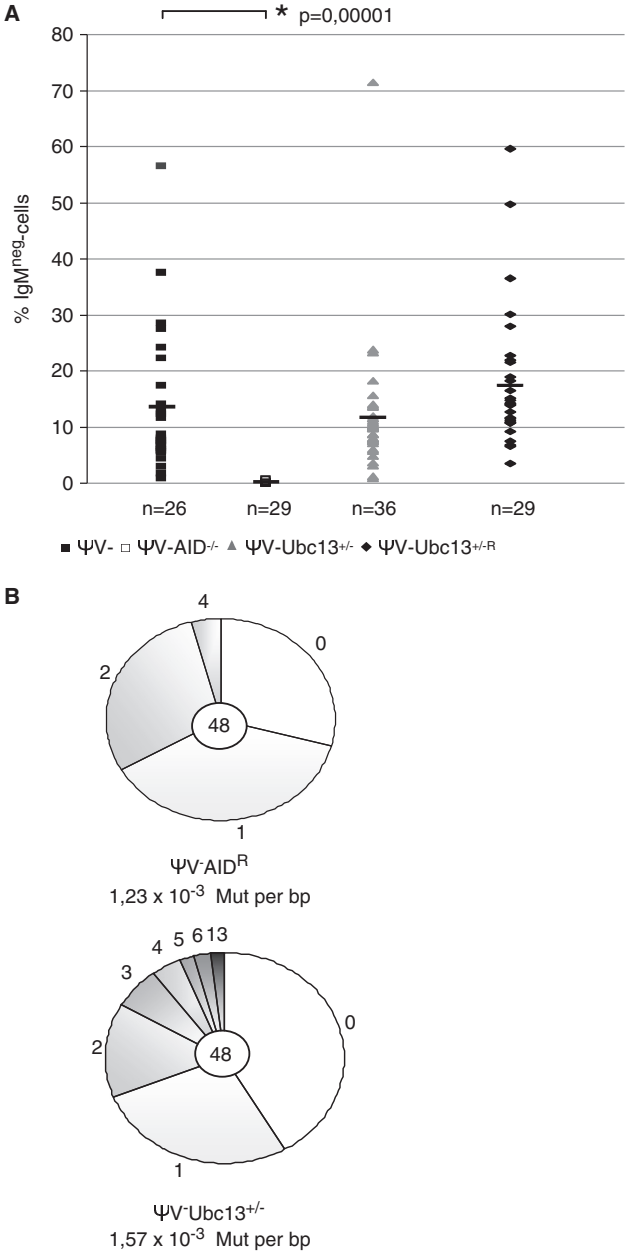

Figure 3. Effects of Ubc13 dosage on SHM. (**A**) Percentage of sIg− cells in individual single-cell clones of the indicated genotypes. The total number of clones analysed is given below the respective genotype. *P*-values for ψV- compared to ψV- Ubc13$^{+/-}$ and ψV- Ubc13$^{+/-R}$ were 0.56002 and 0.77197, respectively. (**B**) Sequence analysis of the rearranged light chain gene in the clones analysed in (A). The relative fraction of sequences with the indicated number of mutations per sequence is given. Total numbers of sequences are shown in the insets, and the mutation frequency is given below the genotype.

substantial increase in Ig GC found in NHEJ DT40 mutants implies that double-strand break intermediates indeed occur during Ig GC (18). We thus reasoned that in Ubc13 heterozygous cells, NHEJ might limit the HR-mediated repair of AID-induced damage at DNA double-strand breaks, and leave no trace in the variable region sequences due to the intrinsically, mostly error-free nature of this process.

Table 2. Targeting efficiencies in WT versus Ubc13$^{+/-}$ clones

Targeted loci	Rad18	RDM1
DT40 Cre1	18/45 (40%)	29/100 (29%)
Ubc13$^{+/-}$ clone 1	5/45 (11%)	18/100 (18%)
Ubc13$^{+/-}$ clone 2	3/45 (7%)	16/100 (16%)

HR and NHEJ are also the two main competing processes determining whether a transfected DNA construct integrates into the genome at a homologous location or at random sites, respectively. Indeed, the futility of direct inactivation attempts of the second Ubc13 allele in our experiments, which has also been observed in a previous study, may point to a targeting defect in Ubc13$^{+/-}$ cells, as Ubc13$^{-/-}$ DT40 cells are in principle viable (11). To obtain insight into the precise nature of the defect in Ubc13$^{+/-}$ cells from a different perspective, we directly assessed targeting efficiency for the Rad18 and RDM1 loci (12,19). In both cases, a significant decrease in targeted integration was detected in Ubc13$^{+/-}$ cells (Table 2, $P < 0.05$ with Fisher's exact test), indicating indeed that a decreased Ubc13 activity impairs HR at the cost of NHEJ in DT40 cells.

DISCUSSION

Most cellular DNA repair pathways are very robust, and minor changes in the activity of core factors have only rarely been reported to affect their efficiency. Ubc13 is a highly unique protein in the cell, though, as it is the only enzyme capable of synthesizing non-canonical ubiquitin chains via K63 linkages. In models assessing its signalling functions, gene-dosage effects have already been seen (20). In the present study, we report that reduction of Ubc13 levels substantially affects Ig GC and targeted DNA integration. Both reactions require efficient HR, and a key function of Ubc13 in the initiation of HR has been revealed before (11). Since HR protects cells from genetic aberrations, one may infer that under certain conditions even moderate perturbations of Ubc13 regulation might challenge the genetic integrity of cells. Presently, we have no evidence that a change in Ubc13 levels alters PCNA polyubiqintination. A detailed study of the E3 enzymes required for PCNA polyubiquitiation is thus mandatory.

A primary mode of Ubc13 regulation is control of its cellular localization. In the cytoplasm of higher eukaryotes, it performs important functions in signalling processes in association with Uev1A (21). In the nucleus, Ubc13 in complex with Mms2 initiates the error-free arm of the Rad6 pathway by PCNA polyubiquination (9,10) and also plays a crucial role in the initiation of HR and other processes at DNA double-strand breaks in association with RNF8 and RNF168 (11,22,23). Moreover, the cellular localization of Ubc13 may be regulated by DNA damage (24), and in vertebrates Ubc13 regulates and is regulated by p53 function (25). Thus, the dosage effect we observe may be a consequence of cross-competition

of the various cellular pathways that require and affect Ubc13. Notably, mere assessment or increase of Ubc13 levels in the cell may not predict or affect its activity in a given process. Accordingly, overall Ubc13 levels do not differ substantially between cell lines that differ in their HR capacity (Supplementary Figure S2).

An intriguing observation is the selectivity of the dosage effects detected in our study. We see compromised Ig GC and targeted DNA integration, both of which depend on efficient HR. However, we do not see effects on overall survival of exogenously caused DNA damage, even for drugs that require efficient HR for optimal tolerance. This apparent discrepancy might be based on the induction of other repair pathways by an exogenously caused multitude of DNA lesions, which might not occur in case of low-dose endogenous damage. However, Ubc13$^{+/-}$ cells can even deal with the rare endogenous challenges during replication, which also require HR for resolution (8), to an extent that allows the cells to proliferate normally. Accordingly, different repair situations may simply be differentially sensitive to changes in Ubc13 levels, potentially based on the relative degree to which they compete with processes less adequate for proper resolution of the lesion. Conversely, one may conclude that an increase of K63-linked ubiquitin conjugation by other means, such as treatments that increase nuclear Ubc13 function or by interference with the competing ubiquitin hydrolase complex Rap80–BRCC36 (26), may shift the balance in favour of exactly those processes that are most sensitive to gradual interference with Ubc13 levels.

In this context, the interference of Ubc13 dosage changes with the efficiency of targeted DNA integration is of particular interest. Targeting efficiencies are exceptionally high in DT40 cells (27), and comparable efficiencies in targeted genetic changes in embryonic or adult stem cells would substantially improve the chances of minimally obstructive gene therapy. It will thus be highly interesting to assess targeting efficiency in mammalian cells, in which the functionality of enzymes regulating Ubc13 function or the turnover of K63-linked ubiquitin chains are modulated.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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