Transient cold shock enhances zinc-finger nuclease–mediated gene disruption

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Zinc-finger nucleases (ZFNs) are powerful tools for editing the genomes of cell lines and model organisms. Given the breadth of their potential application, simple methods that increase ZFN activity, thus ensuring genome modification, are highly attractive. Here we show that transient hypothermia generally and robustly increased the level of stable, ZFN-induced gene disruption, thereby providing a simple technique to enhance the experimental efficacy of ZFNs.

Designer restriction enzymes based on zinc-finger nucleases (ZFNs) have been used to generate functional gene knockouts in organisms as diverse as nematodes, flies, fish, rats and plants, as well as in rodent and human cells[1–10]. This flexibility is driven by the ability to engineer the zinc finger DNA–binding domain to target a ZFN to virtually any DNA sequence and the evolutionary conservation of the DNA repair pathways acting on ZFN-induced DNA double-stranded breaks. Simple methods that could be broadly applied to increase the cellular activity of a ZFN pair across a variety of experimental settings would facilitate application of this promising technology. To this end, much effort has been devoted to developing general strategies for improving ZFN function, including the design of new interdomain linkers and heterodimeric variants of the FokI dimerization interface[11–13]. Here we describe the effect of mild hypothermia (incubation at

30 °C, hereafter referred to as cold shock) on ZFN-driven gene disruption in primary and transformed cell lines.

Our interest in testing whether a transient cold shock would influence ZFN activity in mammalian cells was driven by yeast studies of ZFN efficacy[6]. Specifically, a series of variant ZFN constructs with reduced activity in mammalian cells revealed high levels of ZFN-cleavage activity in yeast reporter assays performed at 30 °C (data not shown). To determine whether an incubation at lowered temperature would improve ZFN-driven gene disruption in human cells, we transfected K562 cells with expression vectors encoding ZFNs targeting the major integration site of the human adeno-associated virus[14] (denoted *AAVS1*) and incubated them at 30 °C and 37 °C. We quantified the introduction of small insertions and deletions (indels), a characteristic signature of ZFN-induced imprecise repair by nonhomologous end joining, using the Surveyor nuclease (Cel-1; Transgenomic) assay[4,12]. At all ZFN concentrations tested, we observed a marked improvement in activity (**Fig. 1a**), an effect independent of the cell line because we also observed it in HeLa cells (**Fig. 1b**). Thus, ZFN-induced mutagenesis of the *AAVS1* locus was enhanced by incubating cells under mild hypothermic conditions.

As the DNA recognition helices of the *AAVS1* ZFNs have been highly optimized, we tested a moderately active ZFN pair known to exhibit suboptimal behavior in cell lines. This ZFN pair has low activity (percentage of indel yield) and causes instability of the modified cells over time in culture. We transfected HeLa cells with plasmids encoding ZFNs targeting the *KDR* gene, split them into three populations for culture under various cold-shock conditions and determined the percentage of indels 3 d later. Under all conditions, the cold shock increased the apparent ZFN activity by two- to tenfold (**Fig. 1c**). Notably, after allowing 21 cell doublings under normal growth conditions (37 °C), up to ~25% of the chromatids were modified in the cold shock–treated population as compared to 1% in cells incubated at 37 °C (**Fig. 1d**). This demonstrates that

Figure 1 | Cold shock enhances ZFN activity in human cells. (**a,b**) Cel-1 assays[4,12] to determine the frequency of indels in K562 cells (**a**) and HeLa cells (**b**) transfected with either 0.5 μg of GFP expression plasmid (−) or indicated amounts of *AAVS1* ZFN expression vectors, divided and incubated at 30 °C or 37 °C for 4 d (**a**) or 3 d (**b**). The asterisks denote underestimated values owing to saturation of the assay[4]. (**c**) Cel-1 assays in HeLa cells transfected with plasmid encoding GFP (*GFP*), 0.4 μg of plasmid encoding CMV promoter–driven *KDR* ZFN (no intron, "−") or 0.4 μg of *KDR* ZFN expressed from a CMV β-globin intron–containing vector ("+"). Immediately after transfection, cells were divided and incubated for 3 d at 30 °C, 1 d at 37 °C followed by 2 d at 30 °C (37,30) or 3 d at 37 °C. (**d**) Cel-1 assays, as in **c**, after transferring the cells to 37 °C for 21 population doublings.
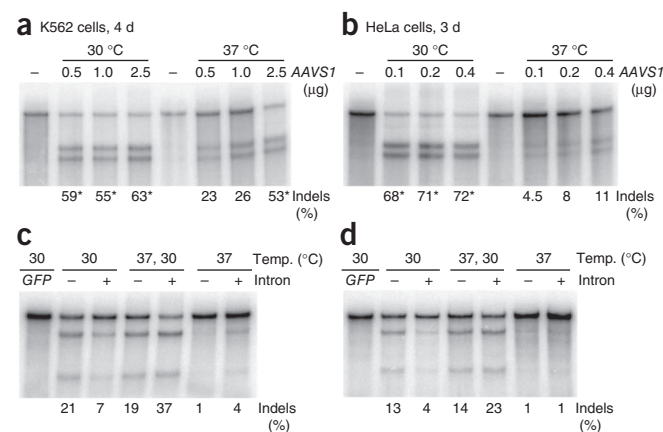
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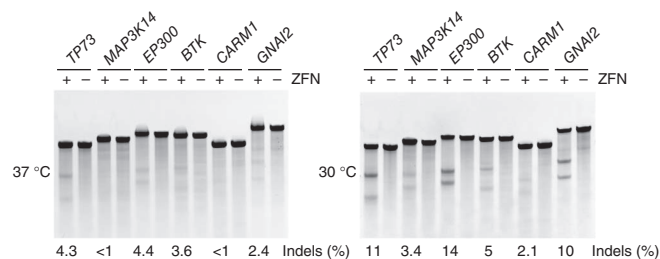
Figure 2 | Effect of the cold shock on ZFNs targeting six genes. K562 cells were transfected with 0.4 µg of ZFN expression vectors and incubated for 3 d at 37 °C (left) or 1 d at 37 °C followed by 2 d at 30 °C (right). The frequency of indels was assessed by the Cel-1 assay[4,12].

the increase in ZFN activity observed immediately after cold shock did not affect long-term survival of the modified cells.

Next, we tested nine different ZFN pairs targeting unique genes. Cold shock resulted in improved gene disruption for every ZFN (**Fig. 2** and **Supplementary Fig. 1**). Notably, ZFN pairs for which we could not detect activity at 37 °C were sufficiently active at 30 °C to provoke a robust gene disruption signal (**Fig. 2** and **Supplementary Table 1**). Cold shock also increased ZFN activity at submaximal DNA vector doses, with the increase in Cel-1 signal remaining stable over time in culture (**Supplementary Figs. 1** and **2**). Cold shock resulted in similar improvements in primary cells as well as transformed lines derived from a variety of species, independent of the ZFN pair or delivery method (**Supplementary Table 1**). The optimal treatment duration varied among different cell lines (**Supplementary Figs. 3** and **4**). Notably, cold shock induced growth arrest in all cell lines tested and a variable but transient growth delay when we returned cells to 37 °C. We conclude that subjecting cells to a cold shock can enhance ZFN-mediated gene disruption of different target genes in different cell types and species.

To begin to evaluate potential mechanisms, we determined both ZFN protein amount and activity under normal and cold-shock conditions. Using ZFNs targeting the *KDR* gene (**Fig. 1c,d**), we observed a dose-dependent and stable augmentation of Cel-1 signal with increasing vector concentration, an effect enhanced by the use of an intron-containing vector at 37 °C (**Supplementary Figs. 3a,b** and **4a,b**). Western blotting revealed a marked increase in the steady-state amount of ZFN protein under all cold-shock conditions tested, in parallel with the improvement in gene disruption efficiency in both HeLa and K562 cells (**Supplementary Figs. 3** and **4**). Thus, the increase in ZFN activity obtained via a transient cold shock resulted, at least in part, from the accumulation of ZFN protein.

To examine whether increased ZFN protein amounts alter cleavage specificity we determined the ratio between on-target and off-target cleavage for a ZFN targeted to the human glucocorticoid receptor gene (*NR3C1*, also known as *GR*), for which two off-target cleavage sites are known in K562 cells (both in noncoding regions of the genome). Using ZFNs containing the wild-type FokI domain, higher amounts of ZFN protein (via cold shock) resulted in a proportional increase in ZFN activity at the intended (*GR*)

site and both off-target sites (**Supplementary Fig. 5**). In contrast, the increase in off-target modification was markedly lower, yet on-target modification very high, when obligate heterodimer FokI variants[12] were used with cold shock (**Supplementary Fig. 5**). Thus, the use of cold shock in combination with obligate heterodimeric FokI domains was an ideal method for achieving high ZFN activity and specificity.

In summary, mild hypothermia enabled reproducible, efficient and stable ZFN-driven genome editing in different cells and species, and using different ZFNs. Several known responses to cold temperatures could contribute positively to this phenomenon, such as a global decrease in mRNA and protein turnover or induction of molecular chaperones. Additionally, enhanced viability and delayed apoptosis might protect modified cells[15]. Our data support ZFN protein levels as at least one potential mechanism for cold shock–driven improvement in ZFN-mediated genome editing. This is a simple and general way to greatly improve ZFN activity *in vivo*, which members of any laboratory can easily integrate into their studies.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

AUTHOR CONTRIBUTIONS
Y.D., V.M.C., D.F.X. and T.D.V. performed the experiments. Y.D. and M.C.H. designed the experiments. Y.D., M.C.H. and P.D.G. wrote the paper.

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ONLINE METHODS

Cell culture and transfection. K562 and HeLa cells were obtained from the American Type Culture Collection and maintained at 37 °C under 5% CO_2 in either RPMI or DMEM media supplemented with 10% FBS. K562 cells were transfected using the 96-well Nucleofector kit SF (Lonza) with the exception of the experiment shown in **Figure 1a**, in which we used the single cuvette format Cell Line Nucleofector Kit V (Lonza) as per the manufacturer recommendations. HeLa cells were transfected using the 96-well Nucleofector kit SE (Lonza). For cold-shock treatments, the incubator was set at 30 °C and 5% CO_2.

ZFN reagents. All plasmids encoding ZFNs were obtained from Sigma-Aldrich, with the exception of the *GR* ZFNs, and used the FokI obligate heterodimer[12] variants unless specifically noted.

Surveyor nuclease (Cel-1) assay. Genomic DNA was extracted from cells using the QuickExtract DNA Extraction solution (Epicentre Biotechnologies). ZFN targeted loci (~300 bp) were PCR-amplified (30 cycles, 60 °C annealing, 30 s elongation) using the oligonucleotides described in **Supplementary Table 2**. The assays were carried out as described previously[4,12].

Western blot. A million cells were lysed directly in 100 µl of loading buffer by boiling. The antibody to Flag M2 (Sigma) was used at 1:1,000 and the antibody to NFκB p65 (Santa Cruz) at 1:3,000.