Supplementary Figure S1

**SD1 and SD2 network of interactions.** A. Interactions in the presence of the nascent cognate base-pair. Polar contacts are represented as dotted red lines, hydrophobic contacts as grey dotted lines. For clarity only one water molecule is represented (W1) in this view. B. Definition of SD1 and SD2 regions. The Substrate Specific Sequence Determinant SD1 and SD2 two regions can be identified by computing a score based on the mutual information based on the multi-alignment of the sub-group made of pol mu (15) sequences versus the sub-group made of Tdt (23) sequences. C and D. View of the water network around the nascent and MH-bp region in the complex where MH-bp is C-C. C. Electron density for W1, W3a and W3b. D. Electron density for W2, W4a and W4b. Loop1 is colored in magenta and SDR1 is in orange.
**Supplementary Figure S2**

**Loop1 and SDR1 conformation in three different structures of Tdt DSB complexes (wild-type and mutants).** In wild type Tdt, Loop1 is well ordered and constrains the MH base pairing even in absence of Watson-Crick hydrogen bonds. In the mutant F405A Loop1 is ordered only if the MH base pair is a cognate one (C-G). However, due to the lack of stabilization of the 3’ sugar ring by the aromatic side chain, the base at the 3’-end of the primer is not visible in the density, but is represented in transparent sticks here. Loop1 is disordered in the mutant F401A and can’t constrain well the MH-bp. The experimental electron densities of Loop1 and the SDR1 are depicted in light blue and contoured at 1σ. The bases that are not well defined in the density are represented in the transparent mode. Loop1 is colored in magenta and SDR1 is in orange.
dNTP incorporation by Tdt in the presence of various in trans templating bases. **A.** Time course addition of one ddNTP nucleotide by wild-type Tdt on different DNA substrates, with a downstream duplex containing Watson-Crick or non Watson-Crick MH base pairs, using various ddNTPs as the incoming nucleotide. The radio-labeled strand is indicated by a star. **B.** Structures of the intermediates in the catalytic cycle of Tdt with a single-stranded primer substrate, adapted to accommodate the DNA DSB substrate. In the post-translocation state the L398 and F405 side-chains have moved away to allow the translocation.
Supplementary Figure S4

Activity of wild-type and mutants of murine Pol mu on different DNA substrates. A. Loop1 and SDR1 sequence alignment. Strictly conserved residues are shown with a red background, similar residues are in red. The numbering corresponds to murine Pol mu (top) and murine Tdt (bottom). Loop1 is represented with a grey rectangle and the SDR1 is in purple. B. Activity of wild-type Pol mu with a Watson-Crick (cognate) or non-Watson-Crick (non cognate) in trans MH base pair (in blue), using either 1 mM MgCl₂ or MnCl₂ in the reaction buffer. The expected dNTP to be incorporated and the in trans templating base are in red. C-D-E. Pol mu mutants’ activity with three different DNA substrates. C. Activity of M384A mutant. D. Activity of F391A mutant. E. Activity of D385E mutant.
Supplementary Figure S5

Functional tests of Helix N Tdt mutants. Mutants were chosen at important positions in the network of interactions around the MH- and nascent base pairs, in Helix N: R454A, R458A and R461A.