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# Monoclonal antibody generation by controlled immunoglobulin gene rearrangements



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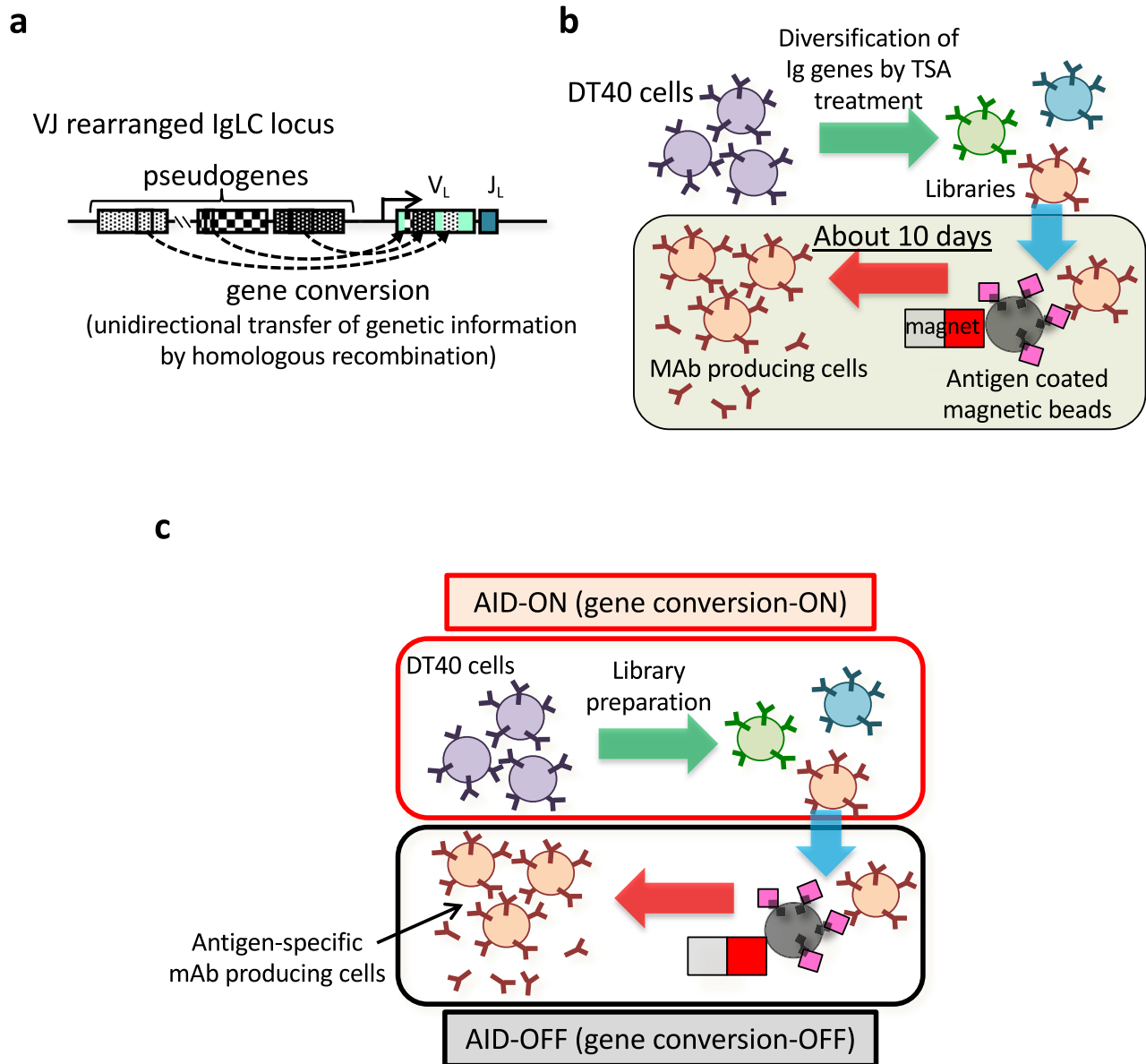
Monoclonal antibodies (mAbs) are essential for various applications including experimental reagents, diagnostics, and therapeutics. Thus, the platform technologies that stably generate antigen-specific mAbs are increasingly crucial. We previously developed a method to generate mAbs, termed the “ADLib system”, utilizing the avian-derived B cell line DT40. Avian immunoglobulin (Ig) genes diversify principally through gene conversion—a kind of homologous recombination. The ADLib system isolates antigen-specific clones from libraries constructed using DT40 cells treated with Trichostatin A (TSA), a histone deacetylase inhibitor that enhances gene conversion frequencies. The obtained antigen-specific clones are cultured without TSA to minimize further diversification. However, low-frequency spontaneous gene conversion still occurs, potentially leading to gradual changes in the specificity of the clones. To address this, we engineered conditional mutants of activation-induced deaminase (AID), the initiator of gene conversion, using auxin-inducible degron system which enables targeted protein degradation via the auxin-dependent ubiquitin-proteasome pathway. The addition of the phytohormone auxin led to the degradation of degron-tagged AID proteins, effectively halting gene conversion. Subsequently, we carried out the ADLib system using these clones and successfully isolated antigen-specific mAbs. These suggest that our AID conditional mutants provide a powerful tool for generating and stabilizing antigen-specific clones isolated by the ADLib system.

In recent years, mAbs have become indispensable in various fields, making the generation of high-quality mAbs a critical priority. While hybridoma method has been the foundational technique for mAb generation<sup>1</sup>, this animal-based technology has bottlenecks of immunological tolerance, where evolutionarily conserved antigens exhibit low immunogenicity, making it difficult to generate mAbs against them. Recently, the use of laboratory animals is restricted by regulations such as Animal Welfare Act, hence it has been getting harder to sacrifice many laboratory animals for mAb generation. To overcome these drawbacks, several *in vitro* methods have emerged, such as phage, yeast, and ribosome display etc<sup>2–4</sup>. Although these *in vitro* methods offer valuable solutions to generate mAbs against antigens of low immunogenicity, they also present challenges in terms of their intensive labor requirements and the extended timeframes needed.

In our previous research, we developed a rapid *in vitro* method for generating mAbs using the avian derived B cell line, DT40<sup>5–8</sup>. Human or murine pre-immune repertoire of Ig gene are generated via V(D)J recombination, whereas the avian Ig genes are diversified mainly through gene conversion<sup>9–11</sup>. This gene conversion process involves the overwriting of functional variable (V) regions by partial sequences derived from upstream pseudogenes which show homologies to the functional V region (Fig. 1a). Interestingly, this is a unidirectional event wherein the functional V region is altered, while pseudogenes remain unchanged. DT40 cells are unique in several respects: 1) they exhibit gene conversion at their Ig loci, albeit at a low frequency; 2) they express both membrane-bound and secreted versions of IgM protein; 3) they exhibit rapid growth, with a doubling time of approximately 8 h; and 4)

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**Fig. 1 | The ADLib system using AID conditional mutant. a** Diversification of avian Ig genes by gene conversion. The functional  $V_L$  is overwritten by partial sequences from upstream pseudogenes via homologous recombination.  $J_L$  indicate the junctional region of LC. **b** Principle of the ADLib system. DT40 cells are cultured with TSA to generate diversified cell-based mAb libraries. Antigen-specific clones can be isolated by, for example, antigen-coated magnetic beads. The isolated cells are

expanded, and the antigen-specific mAbs are recovered in culture supernatants. **c** Schematic representation of the ADLib selection using AID conditional mutant. First, library is prepared by turning on the expression of AID, which induces gene conversion (upper layer). Next, mAb selection is performed after turning off the expression of AID, thereby stabilizing the isolated antigen-specific clones (lower layer).

they allow ease of target integration<sup>12–14</sup>. We discovered that the treatment of DT40 cells with TSA markedly enhances the gene conversion frequency, which is possibly mediated through alterations in the chromatin structure at the Ig loci<sup>5</sup>. Leveraging this phenomenon, we developed cell-based antibody display libraries through autonomous diversification by gene conversion, a method we term the Autonomously Diversifying Library (ADLib) system<sup>5,6</sup>. Remarkably, this system can generate mAbs in roughly 10 days in the shortest time (Fig. 1b) using simple cell culturing and magnetic bead selection, followed by screening such as enzyme-linked immuno-sorbent assay (ELISA).

To date, this original ADLib system has yielded mAs with biological functions<sup>15–17</sup>, and three diagnostic antibodies have entered the market including “Lumipulse G 25-OH Vitamin D”<sup>18</sup>, “Lumipulse Presto Aldosterone”, and “Lumipulse Presto iTACT” by Fujirebio Inc. Moreover, we have successfully developed humanized version of the ADLib system

(human ADLib system) in which chicken Ig genes and pseudogenes of DT40 cells are replaced by human counterparts<sup>19</sup>. The human ADLib system enables the initial acquirement of antigen-specific human IgG mAbs, which are more suitable for therapeutic purposes.

Since the TSA-induced enhancement of gene conversion is reversible, removal of TSA from the medium decreases gene conversion frequency, but it continues at a low rate<sup>5</sup>. This raised a concern that post-selection culturing could gradually alter the specificities or affinities of selected antigen-specific clones. To address this issue, we engineered DT40 cells to allow for controllable gene conversion activities. We focused on AID, a cytidine deaminase initially identified as the trigger of somatic hypermutation Ig class switch recombination<sup>20</sup>. Multiple studies have shown that AID also functions in gene conversion at Ig loci. Notably, Arakawa et al. and Harris et al. reported that AID knockout in DT40 cells effectively halts gene conversion<sup>21,22</sup>. We reasoned that conditional knockout of AID after

antibody selection by the ADLib system could inhibit gene conversion activity, thereby stabilizing the selected antigen-specific clones (Fig. 1c).

To that end, we initially developed a method to efficiently monitor gene conversion using fluorescent proteins, since measuring gene conversion frequency is a crucial step in this work, and conventional immune staining-based methods are rather time-consuming. With this method, cells that originally fluorescence negative turn to express fluorescence protein when gene conversion occurs at Ig locus, allowing to observe gene conversion events without immuno-staining simply by flow cytometer.

For conditional AID depletion, we employed the auxin-inducible degron system, which utilizes plant-derived protein degradation via the ubiquitin-proteasome pathway (Supplementary Fig. 1)<sup>23</sup>. In the auxin-inducible degron system, rice-derived ubiquitin ligase TIR1 induces degron protein degradation in the presence of the plant hormone auxin. Multiple DT40-based conditional mutants have been constructed using this system to date<sup>24–28</sup>. We introduced TIR1 expression vector and degron-tagged AID expression vector into AID-knocked-out DT40 cells. In these cells, gene conversion at Ig loci occurred at moderate levels even in the absence of TSA treatment. We confirmed that the addition of auxin to the medium led to the depletion of degron-tagged AID proteins, abolishing gene conversion. Furthermore, by culturing these AID conditional mutants without auxin, we constructed DT40 cell-based antibody display libraries and successfully generated specific mAbs against multiple antigens. In summary, we propose that these AID conditional mutants facilitate both the generation and stabilization of antigen-specific mAbs.

Since the auxin-inducible degron system is also abbreviated as “AID”, we have named these conditional mutants the “AID<sup>2</sup>” (AID square). However, for clarity, we will use in this paper “AID” solely to designate activation-induced deaminase.

## Results

### Development of a method to monitor gene conversion frequency using fluorescence protein

Before constructing the conditional mutant of AID, we first developed a method to monitor gene conversion frequency using fluorescence proteins. Conventionally, gene conversion frequencies have been measured by a reversion assay<sup>13</sup>. In this assay, clones that have turned surface IgM negative (sIgM<sup>−</sup>) due to the introduction of a frameshift mutation at the Ig light chain (LC) variable region (V<sub>L</sub>) can revert to IgM positive (sIgM<sup>+</sup>) through gene conversion, which repairs the frameshift mutation (Fig. 2a). Standard reversion assays involve staining DT40 cells with fluorescence-labeled anti-IgM antibodies, followed by fluorescence activated cell sorter (FACS) analysis. To acquire statistically reliable results, fluctuation analysis is performed, necessitating the examination of many subclones (~24) for each strain<sup>21,29,30</sup>.

To simplify the reversion assay, we employed a fluorescence protein, mCherry, as a reporter. The mCherry gene was linked to the constant region of the chicken LC gene via a P2A self-cleavage sequence<sup>31</sup>. Additionally, a frameshift mutation (single nucleotide insertion) was introduced into the complementarity determining region 1 (CDR1) of the V region. Theoretically, repair of the frameshift mutation by gene conversion results in sIgM<sup>+</sup> and mCherry<sup>+</sup> cells (Fig. 2b), allowing for the monitoring of gene conversion events by fluorescence. We constructed the knock-in vector containing the components required for this method and integrated it to the LC locus of DT40 (Supplementary Fig. 2a). After transfecting this construct and subsequently culturing the bulk population with puromycin, we identified three major cell populations in FACS profile: sIgM<sup>−</sup>/mCherry<sup>−</sup>, sIgM<sup>+</sup>/mCherry<sup>+</sup>, and sIgM<sup>+</sup>/mCherry<sup>−</sup> (Supplementary Fig. 2b). Since the cells that the vector was properly knocked-in are predicted to express neither sIgM nor mCherry, we isolated sIgM<sup>−</sup>/mCherry<sup>−</sup> cells by single-cell sorting and confirmed that each clone was basically mCherry<sup>−</sup> but contained some population of mCherry<sup>+</sup> cells, likely resulting from gene conversion (Supplementary Fig. 2c).

We selected a clone with the highest mCherry<sup>+</sup> ratio (Supplementary Fig. 2c) and confirmed proper targeting of the knock-in vector through

genotyping PCR (Supplementary Fig. 2d). The drug resistance gene was subsequently removed by transient expression of Cre recombinase (Supplementary Fig. 2e), yielding the representative clone, referred to as mp232. Sequence analysis of the V<sub>L</sub> of mp232 cells revealed a single nucleotide insertion identical to that in the knock-in vector. Similarly to the parental clone shown in Supplementary Fig. 2c, mp232 contains sIgM<sup>+</sup>/mCherry<sup>+</sup> cells possibly caused by gene conversion. We analyzed the sequences of V region of the sorted sIgM<sup>+</sup>/mCherry<sup>+</sup> cells. As expected, we found that the frameshift mutation in the V<sub>L</sub> was repaired by partial sequences corresponding to pseudogenes (Fig. 2c). These data suggests that the sIgM<sup>+</sup>/mCherry<sup>+</sup> cells are generated via gene conversion. We also compared the ratio of mCherry<sup>+</sup> cells of mp232 and IgM<sup>−</sup> cells of CL18 (a previously reported sIgM<sup>−</sup> DT40 clone which harbors the identical single nucleotide insertion in V<sub>L</sub>) by fluctuation analysis and found that these two clones are equivalent (Fig. 2d).

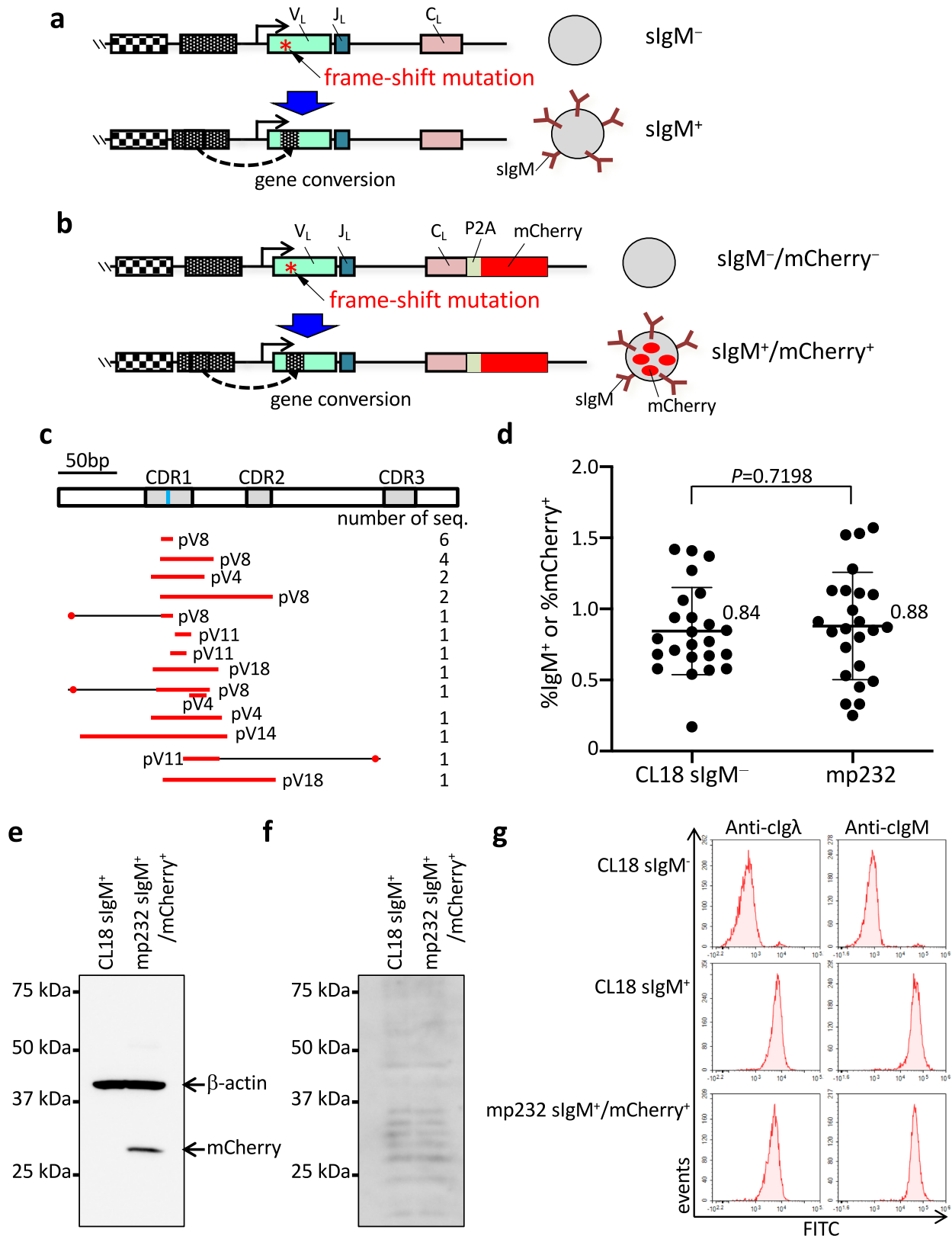
We then verified that in sIgM<sup>+</sup>/mCherry<sup>+</sup> mp232 cells, the P2A sequence effectively mediated the separate expression of the chicken LC and mCherry proteins as distinct entities. Immunoblot analysis revealed that mCherry protein is expressed as a discrete protein, showing the predicted molecular weights (26.7 kDa) (Fig. 2e). However, chicken LC fused with P2A (24.4 kDa) was not detected by commercially available anti-chicken LC antibody (Fig. 2f). Since signal obtained using anti-mCherry was a single band corresponding to the molecular weight of mCherry, un-cleaved “LC-P2A-mCherry” protein (51.1 kDa) was likely to be not expressed. Moreover, we successfully detected chicken LC protein expressed on the surface of the cells by flow cytometer using the identical anti-chicken LC antibody to the one used in immunoblot (Fig. 2g). From these, we consider that chicken LC and mCherry are translated as discrete protein. Taken together, we concluded that mp232 cells can be utilized as monitoring system for tracking gene conversion frequency and parental cells for the construction of AID<sup>2</sup> cells.

### Construction of AID<sup>2</sup> cells

Next, we constructed AID<sup>2</sup> cells according to the scheme described in Fig. 3a. Initially, the AID genes of mp232 cells were homogeneously disrupted. Subsequently, the expression vector for TIR1 ubiquitin ligase was introduced into the AID-knockout cells by random integration. This was followed by the transfection of degron-tagged AID expression vectors to obtain AID<sup>2</sup> cell candidates. AID knockout was performed through targeted disruption and confirmed by genotyping PCR as described in Arakawa et al.<sup>21</sup> (Supplementary Fig. 3a–d). AID transcripts were not detectable by semi-quantitative RT-PCR (Supplementary Fig. 3e), showing that AID genes were knocked out successfully. We further examined the gene conversion frequency in AID-knockout mp232 (mp232-ΔAID) cells by fluctuation analysis and found that mCherry<sup>+</sup> cells did not emerge, indicating that gene conversion is abolished, consistent with previous reports<sup>21,22</sup> (Fig. 3b).

Next, we ectopically introduced a cMyc-tagged TIR1 expression vector to the mp232-ΔAID cells. TIR1 expression was assessed through immunoblotting using anti-cMyc-tag antibodies. Since TIR1 is reported to induce leaky degradation without auxin<sup>32</sup>, we selected a clone (#2 in Supplementary Fig. 4a) with a modest expression level of TIR1 among the candidates. We then ectopically introduced degron-tagged AID expression vectors. Both C-terminal and N-terminal ×3 degron-tagged AID (AID-degron and degron-AID respectively) were evaluated (Fig. 3c). However, as AID contains a nuclear localization signal (NLS) at its N-terminal region, we were afraid that direct addition of a degron tag to their N-terminus might perturb the NLS function. To mitigate this, we constructed a third version of degron-tagged AID, in which the degron tag was inserted downstream of the NLS (NLS-degron-AID (ΔNLS)) (Fig. 3c). In addition to chicken AID (GdAID), we also used murine AID (mAID).

Flow cytometric analysis of the resulting transfectants revealed that cells transfected with C-terminal degron-tagged GdAID and mAID expression vectors contained mCherry<sup>+</sup> populations (Fig. 3d, e). However, clones transfected with N-terminal degron-tagged AID and NLS-



degron-AID ( $\Delta$ NLS) expression vectors had minimal or no mCherry<sup>+</sup> cells (Supplementary Fig. 4b–e). Given that mCherry<sup>+</sup> cells are generated through gene conversion, we inferred that only the C-terminal degron-tagged AID possessed the capability to induce gene conversion. Thus, subsequent analyses focused on cells harboring this C-terminal tagged version.

We further tested whether C-terminal degron-tagged GdAID or mAID proteins underwent degradation upon the addition of auxin to the medium. Immunoblot analysis using an anti-degron antibody showed that these proteins efficiently degraded within 1 h after the addition of auxin to the culture (Fig. 4a). This suggests successful construction of the AID conditional mutant strains. Then we measured the gene conversion frequencies of



**Fig. 2 | Construction of gene conversion monitoring cells.** **a** Principle of reversion assay. The frame-shift mutation introduced into  $V_L$  abolishes the expression of sIgM (upper). Gene conversion events that repair the frameshift mutation can restore the expression of sIgM (lower).  $C_L$  indicate the constant region of LC. **b** Schematic representation of the reversion assay using mCherry. The frame-shift mutation introduced into  $V_L$  abolishes the expression of both sIgM and mCherry linked to the constant region via P2A (upper). Gene conversion events that repair the frameshift mutation restore the expression of both sIgM and mCherry (lower). **c** Overview of the sequence alteration in  $V_L$  responsible for sIgM<sup>+</sup>/mCherry<sup>+</sup> property. Red lines and circles indicate gene conversion tracts and point mutations, respectively. The corresponding pseudogenes are indicated alongside. The number of sequences identified were shown on the right side. Blue line in CDR1 indicates the position of frame-shift mutation. **d** Comparison of the gene conversion frequency of CL18 (left)

and that of mp232 (right) by fluctuation assay. For the ratio of sIgM<sup>+</sup> cells is shown for CL18 and mCherry<sup>+</sup> cells for mp232, respectively.  $n = 24$  for each experimental group. Long horizontal bars represent the mean, and their values are shown alongside. Error bars represent  $\pm$  s.d. Differences were compared using a two-tailed  $t$ -test. **e** Immunoblot analysis to examine the expression of mCherry in mCherry<sup>+</sup> mp232 cells. Results using anti-mCherry antibody are shown. **f** Immunoblot analysis to examine the expression of Ig $\lambda$  in mCherry<sup>+</sup> mp232 cells. Results using anti-chicken Ig $\lambda$  antibody (right) using identical membrane to **e** are shown. No signal was detected even for parental CL18 sIgM<sup>+</sup> cells. **g** Flow cytometry analysis to examine the expression of chicken LC in mCherry<sup>+</sup> mp232 cells. Cells were stained with anti-chicken Ig $\lambda$  (left) and chicken IgM (right) antibodies labeled with FITC. Results for CL18 sIgM<sup>+</sup> (top), CL18 sIgM<sup>+</sup> (middle) and mp232 mCherry<sup>+</sup> (bottom) are shown.

these AID<sup>2</sup> candidate cells in fluctuation assays, performed with and without auxin (Fig. 4b). In the absence of auxin, murine version AID<sup>2</sup> (mAID<sup>2</sup>) cells (clone mAd1-1 and mAd1-4) exhibited gene conversion frequencies (0.38% and 0.31% of mCherry<sup>+</sup> ratio, respectively) comparable to the original mp232 (0.26%), while the chicken version AID<sup>2</sup> (GdAID<sup>2</sup>) cells (clone gAd2-1 and gAd2-4) demonstrated somewhat lower gene conversion frequencies (0.10% and 0.15%, respectively). In the presence of auxin, gene conversion was markedly abolished in all the AID<sup>2</sup> candidate clones examined ( $2.5 \times 10^{-3}\%$  for gAd2-1,  $1.3 \times 10^{-3}\%$  for gAd2-4,  $3.3 \times 10^{-3}\%$  for mAd1-1 and  $8.3 \times 10^{-4}\%$  for mAd1-4, respectively). Importantly, the gene conversion frequency of two GdAID<sup>2</sup> clones (gAd2-1 and gAd2-4) and one of the two mAID<sup>2</sup> clones (mAd1-4) cultured with auxin is statistically insignificant compared with mp232- $\Delta$ AID (0.00%), suggesting that these cells are equivalent to mp232- $\Delta$ AID when cultured with auxin. There is a statistically significant difference in gene conversion frequencies between one of the mAID<sup>2</sup> cells (mAd1-1) cultured with auxin and mp232- $\Delta$ AID cells. Despite this, since the difference between the mean mCherry<sup>+</sup> ratio of this clone ( $3.3 \times 10^{-3}\%$ ) and mp232- $\Delta$ AID (0.00%) is extremely small, we conclude that all of the four auxin-treated AID<sup>2</sup> clones are practically equivalent to mp232- $\Delta$ AID cells. This outcome suggests that the auxin-inducible degon system functioned effectively in AID<sup>2</sup> cells, enabling to control gene conversion activities.

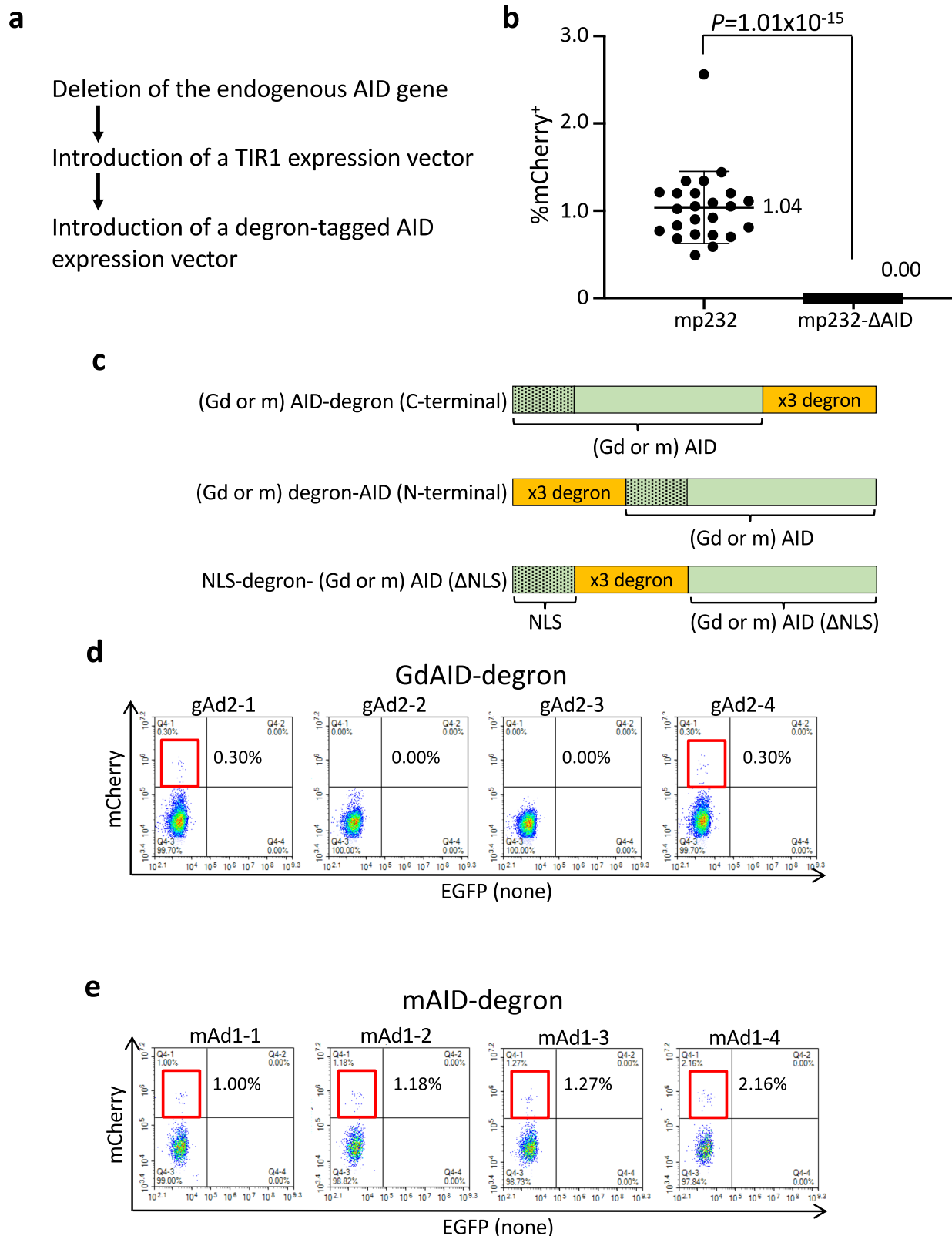
Since expression of mCherry enables monitoring of the rearrangements only in the  $V_L$  region, we next examined whether the addition of auxin inhibits immunoglobulin rearrangements in the  $V_H$  regions. We compared the sequences of the 24 subcloned derived from representative clone (mAd1-4) cultured with or without 1 mM auxin for two weeks (Fig. 4c). In DT40 cells, the AID-dependent somatic hypermutation predominantly occurs at G/C sites, suggesting that sequence alterations at A/T sites are mostly attributed to gene conversion<sup>11,33</sup>. For the auxin-treated cells, we observed 34 somatic hypermutation events at G/C sites and 18 at A/T sites, respectively. Additionally, 11 sequence alterations involving multiple successive nucleotides were observed. As the heavy chain (HC) pseudogene sequences are only partially clarified, we cannot determine whether these alterations are caused by gene conversion or somatic hypermutation. However, given the frequency of point mutations (34 at G/C sites in 24 independent sequences), it is highly probable that they are attributed to somatic hypermutation rather than gene conversion. In summary, 63 sequence alterations were observed for the auxin-treated mAd1-4 cells. For mAd1-4 cells cultured with 1 mM auxin, the number of sequence alterations significantly decreased to four in total (three events at G/C sites and one at A/T sites, with no multiple successive sequence alterations). These indicate that the addition of auxin also inhibits the immunoglobulin rearrangements in HC locus in AID<sup>2</sup> cells.

### Application of AID<sup>2</sup> cells to the ADLib system

We next applied GdAID<sup>2</sup> and mAID<sup>2</sup> cells to the ADLib system. To construct libraries, we cultured the GdAID<sup>2</sup> and mAID<sup>2</sup> clones with or without TSA (Supplementary Fig. 5). These AID<sup>2</sup> clones exhibited higher gene conversion activity when cultured with TSA than without. However, the effect of TSA on AID<sup>2</sup> cells are somehow less pronounced than that on the

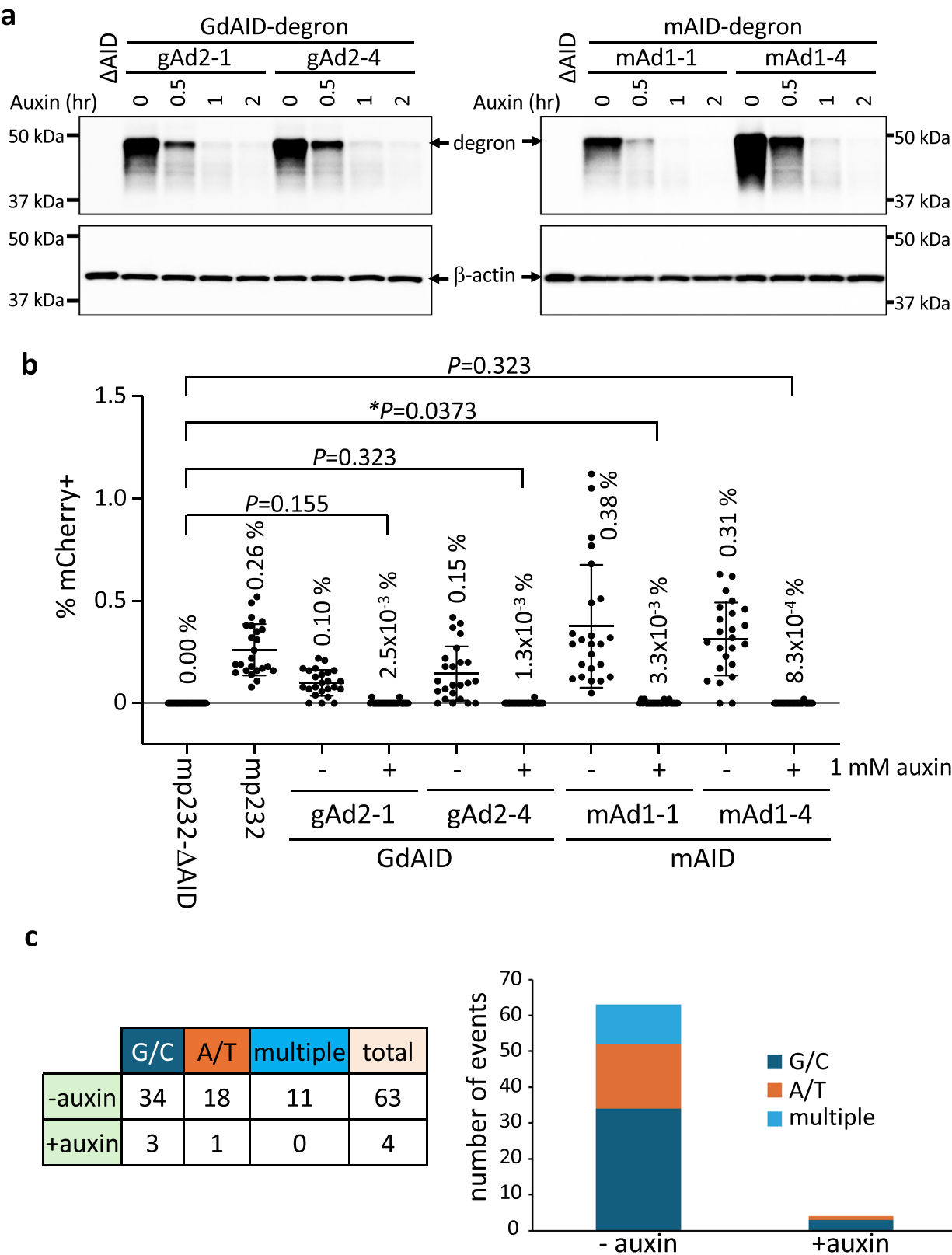
parental mp232 cells, and the difference between TSA-treated and untreated AID<sup>2</sup> cells was smaller than that observed in mp232 cells. Notably, the increase of mCherry<sup>+</sup> cells in the TSA-untreated AID<sup>2</sup> cells from week three to four apparently exceeded that in parental mp232 cells, suggesting that libraries can be prepared without TSA treatment. Consequently, we opted to construct libraries by culturing GdAID<sup>2</sup> or mAID<sup>2</sup> cells without TSA for 6 to 8 weeks followed by the selection using magnetic beads. As a model antigen, we first examined rabbit IgG (rIgG). We conjugated the magnetic beads with rIgG protein and contacted with the libraries. Next, we washed the magnetic beads, and harvested the antigen-specific clones that bound to the magnetic beads using a magnet. These cells were then subjected to limiting dilution in 96-well plates. After one week of culturing, we screened antigen-specific clones by ELISA using culture supernatants. Note that to avoid the gene conversion after magnetic beads selection, we added 1 mM auxin to the buffer for the magnetic beads selection and culture media for limiting dilution. By ELISA, we identified multiple clones that reacted with rIgG but not with ovalbumin, a negative control antigen (Fig. 5a). Interestingly, we obtained multiple anti-rIgG clones from mAID<sup>2</sup> cell derived library, whereas only one positive clone was isolated from GdAID<sup>2</sup> cell derived library. Subsequently, we further examined the specificities of the representative clones that passed the initial ELISA screening. We found that they reacted with rIgG, while they showed no reaction with negative control antigens, suggesting that these clones were highly specific against rIgG (Fig. 5b).

We then tested additional model antigens, namely apoferritin. In these experiments as well, we obtained multiple antigen-specific clones but contrary to the anti-rIgG antibody selection, all of them were obtained from GdAID<sup>2</sup> cell derived library (Fig. 5c). We isolated no antigen-specific clone from mAID<sup>2</sup> cell derived library. We hypothesized that anti-apoferritin clones contained in mAID<sup>2</sup> derived libraries might have failed to be captured by magnetic beads due to the use of only a single round of magnetic beads selection; therefore, we implemented multiple rounds of panning. Initially, the mAID<sup>2</sup> cell derived libraries were incubated with apoferritin-conjugated magnetic beads and bulk-cultured for approximately one week. The cells that were recovered underwent further contact to the antigen-conjugated magnetic beads. After three rounds of panning, the remaining cells were isolated by limiting dilution into 96-well plates. We then assessed the specificity of the subclones by ELISA using culture supernatants, finding multiple clones that reacted with apoferritin but not with ovalbumin (Fig. 5d). We next further examined the specificity of representative clone and showed that it is reactive with apoferritin but not reactive to the negative control antigens (Fig. 5e). Lastly, we carried out the ADLib selection against human epidermal growth factor receptor (EGFR), one of the key targets for therapeutic antibodies. Since EGFR is a membrane protein, we used its extracellular domain fused with human IgG Fc region (EGFR-Fc). Also in this case, we successfully isolated antigen-specific clones in the first screening ELISA both from GdAID<sup>2</sup> and mAID<sup>2</sup> derived libraries (Fig. 5f). We further examined the specificities of the representative clones and found that they reacted with EGFR-Fc but not with negative control antigens (Fig. 5g). Since the used antigen protein was EGFR-Fc, there was a possibility that the obtained antibodies could react with the human IgG Fc region



**Fig. 3 | Construction of AID<sup>2</sup> cells using degron-tagged AID.** **a** Flowchart of the AID<sup>2</sup> cell construction process. First, endogenous AID genes are homogeneously knocked out, then TIR1 expression vector is introduced followed by the transfection of degron-tagged AID expression vector. **b** Comparison of the gene conversion frequency of mp232 (left) and mp232-ΔAID (right). Sample sizes are  $n = 24$  for mp232 and  $n = 23$  for mp232-ΔAID. Long horizontal bars represent the mean, and their values are shown alongside. Error bars represent  $\pm$  s.d. Differences were

compared using a two-tailed *t*-test. **c** Schematic representation of x3 degron tagged AID. C-terminal tagged (“AID-degron”, top), N-terminal tagged (“degron-AID”, middle) and the version degron tag was inserted downstream of NLS (“NLS-degron-AID (ΔNLS)”, bottom). **d, e** Gene conversion activity of the C-terminal degron tagged GdAID (**d**) and mAID (**e**). Results for the representative four independent clones are shown. Population of mCherry positive cells are highlighted by red rectangles. Ratios of the mCherry positive cells (%) are shown alongside.



rather than EGFR. However, as the negative control antigens included human IgG, we concluded that these clones specifically react with EGFR, not the Fc region of human IgG.

To further characterize the properties of the obtained clones, we used competition ELISA to measure the concentration of competitor required to inhibit 50% of the binding of the free antibody in solution (IC<sub>50</sub>). We

analyzed anti-rIgG#1(m) and anti-Apo#4(Gd) and determined their IC<sub>50</sub> values to be 0.47 μM and 6.7 nM, respectively (Supplementary Fig. 6a, b). In our previous work, we reported on anti-apoferritin antibodies obtained from the ADLib system, with an IC<sub>50</sub> value of 1.7 nM<sup>34</sup>, similar to that of anti-Apo#4 (Gd). These findings suggests that the AID<sup>2</sup>-based ADLib system performs equivalent to the conventional ADLib system.

**Fig. 4 | Control of gene conversion activities using AID<sup>2</sup> cells. a** Immunoblot analysis to examine the depletion of degron-tagged AID proteins. The results are shown for two independent clones of GdAID-degion (clones gAd2-1 and gAd2-4) (left) and mAID-degion (clones mAd1-1 and mAd1-4) (right) cultured with 1 mM of auxin for 0, 0.5, 1 and 2 h.  $\beta$ -actin is loading control. **b** Effect of auxin on the gene conversion frequencies of AID<sup>2</sup> candidate cells. Results of gAd2-1, gAd2-4, mAd1-1 and mAd1-4 cultured with or without 1 mM of auxin are shown. The outcomes for the original mp232 and parental mp232- $\Delta$ AID are also presented for comparison.  $n = 24$  for each experimental group. The long horizontal bar in each series represents

the mean, and their values are displayed vertically above. Error bars represent  $\pm$  s.d. Differences were compared using a two-tailed *t*-test. **c** Analysis of V<sub>H</sub> gene rearrangements in mAd1-4 cells with and without 1 mM auxin culturing. Six mCherry<sup>+</sup> subclones from mAd1-4 were cultured for two weeks with or without auxin. Genomic DNA was extracted from each subclone, and the V<sub>H</sub> gene was PCR-amplified, cloned into vector and transformed into bacteria. Colony PCR was then used to amplify V<sub>H</sub> genes from bacterial colonies for direct sequencing. The table (left) and bar charts (right) display the number of point mutations at A/T and G/C, as well as multiple successive sequence alterations.

We then analyzed the nucleotide sequences of the V region of the obtained antigen-specific clones. As to V<sub>L</sub>, we found multiple sequence alterations which are observed more frequently in CDRs than in frame regions (FRs) (Fig. 6a–c). For each of the analyzed V<sub>L</sub> partial sequences we identified that matched with those of pseudogenes, suggesting that gene conversion events occurred. In all the analyzed clones, the frameshift mutation originally introduced to CDR1 of V<sub>L</sub> was repaired, turning their LC genes in-frame. In addition to gene conversion events, we also found point mutations possibly attributed to somatic hypermutation (for example, FR1 of anti-Apo#4 (Gd)V<sub>L</sub> (Fig. 6b) and CDR3 of anti-EGFR#1 (Gd)V<sub>L</sub> (Fig. 6c)).

We also observed multiple sequence alterations in HC V region (V<sub>H</sub>) from the parental AID<sup>2</sup> clones (Fig. 6d–f). Since the sequences of the chicken HC pseudogene have been clarified only partially, we could not determine whether the observed sequence alterations are the consequence of gene conversion. However, multiple nucleotide substitutions observed in relatively short range (for example, CDR1 of anti-rIgG#2(m)V<sub>H</sub> (Fig. 6d) and CDR2 of anti-rIgG#2(Gd)V<sub>H</sub> (Fig. 6d)) suggest that gene conversion, rather than somatic hypermutation, occurred at these regions. In contrast, isolated point mutations observed, in FR1 or FR3 of anti-Apo#4(Gd)V<sub>H</sub>, FR1, CDR1 or CDR2 of anti-Apo#1(m) (Fig. 6e), or FR4 of anti-EGFR#1(m)V<sub>H</sub> (Fig. 6f) might be caused by somatic hypermutation. However, we cannot exclude the possibilities that these single nucleotide variations are the consequence of gene conversion events. Especially, the identical mutations in the V<sub>H</sub> of two independent clones are likely to be the consequence of gene conversion (for example, FR3 of anti-EGFR#1 (Gd) and anti-EGFR#1 (m) (Fig. 6f). These data suggest that antigen specificities were conferred by gene conversion and/or somatic hypermutation in V<sub>L</sub>s and V<sub>H</sub>s. Predicted amino acid sequences revealed that these sequence alterations in nucleotide generate diversity at the amino acid sequence level (Supplementary Fig. 7a–f). Taken together, we conclude that AID<sup>2</sup> cells can be utilized as parental cells for the ADLib system.

## Discussion

In this study, we initially developed a method to monitor gene conversion frequency using fluorescence proteins, specifically mCherry. This method simplifies conventional reversion assays and allows for more efficient quantification of gene conversion frequencies. Based on this method, we then constructed AID<sup>2</sup> cells. This involved the disruption of AID genes followed by the introduction of plant-derived ubiquitin ligase TIR1 and degron-tagged AID expression vectors. We successfully generated AID<sup>2</sup> cell lines in which AID degradation can be controlled by the addition of auxin, allowing precise regulation of gene conversion frequency. These AID<sup>2</sup> cells exhibit gene conversion frequencies comparable to the parental mp232 cells when AID is present and show halted gene conversion frequency at LC locus to the similar level of mp232- $\Delta$ AID cells when AID was degraded by the addition of auxin. Additionally, we confirmed that immunoglobulin rearrangements (somatic hypermutation or gene conversion which cannot be determined) at HC locus are inhibited by the addition of auxin. Finally, we applied these AID<sup>2</sup> cells to the ADLib system. While TSA treatment enhanced gene conversion frequencies of AID<sup>2</sup> cells, the increase in mCherry<sup>+</sup> cells in cell in the TSA-untreated from week three to four was greater than that in parental cells. The reason for this phenomenon is unclear; however, it might be possible that long-term TSA treatment could

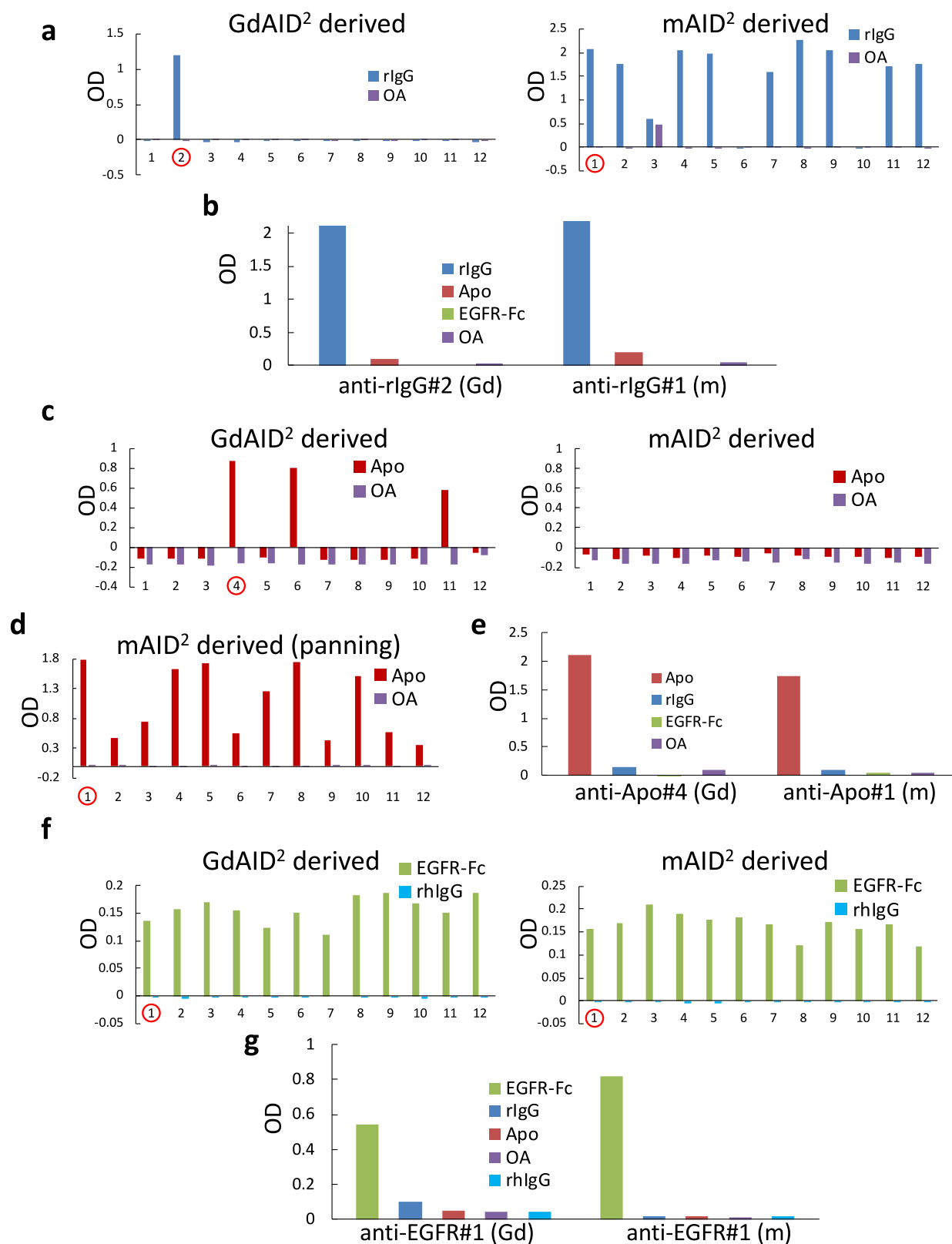
somehow influence the expression level or stability of degron-tagged AID proteins. Therefore, we opted to culture the AID<sup>2</sup> cells without TSA for the construction of libraries. Using these AID<sup>2</sup> based libraries, we effectively generated mAbs with high specificity against various antigens, including rabbit IgG, apoferritin, and EGFR, a therapeutically relevant target. Initially, we failed to isolate anti-apoferritin clones from mAID<sup>2</sup> derived libraries; however, three rounds of panning with these libraries, we successfully obtained apoferritin-specific clones. This suggests that the multiple rounds of panning can be an effective strategy to isolate antigen-specific clones, especially when they are difficult to obtain. Furthermore, the measurement of the binding IC<sub>50</sub> of the representative clones indicates that the AID<sup>2</sup>-based ADLib system can generate antibodies with similar performances to the conventional ADLib system. Sequence analysis of the V regions of the obtained mAbs revealed the alterations consistent with gene conversion or somatic hypermutation, highlighting the potential of AID<sup>2</sup> cells as a powerful tool for generating antigen-specific mAbs.

To date, two approaches have been reported to construct conditional mutant using DT40 cells for the purpose of antibody library construction. For instance, Kanayama et al. generated “DT40-SW cells”, in which the transcription of the AID gene can be switched reversibly using two oppositely oriented loxP sites and a selection marker gene<sup>35,36</sup>. In their conditional mutant, induction of Cre recombinase inverts the AID gene, placing it in the opposite orientation to promoter and transcription of AID halts. Simultaneously, this inversion event places the selection marker cDNA downstream of promoter and thereby drug resistance is conferred, allowing to concentrate the inverted cells by drug selection. This approach enables the complete conditional abolishment of AID transcription, yet there are notable caveats. First, it is necessary to abolish the expression of AID prior to antibody selection; however, drug selection during the inversion process kills the non-inverted cells, which are equivalent to theoretically half of the cells included in the libraries. This can cause a loss of library diversity, which could reduce the possibilities of obtaining antigen-specific clones. Second, proteins and mRNAs of AID expressed before the inversion process are expected to remain in the cells and can induce gene conversion even after inversion is completed until they are degraded. This suggest that even if AID transcription is turned off right before antibody selection, gene conversion still continue after selection, altering the specificity and affinity of the antigen-specific clones.

As the second example of AID conditional mutant, Wang et al. constructed the AID conditional mutant of DT40 based on a tetracycline-inducible expression system<sup>37</sup>. In their conditional mutant, AID is transcribed in the absence of doxycycline while it is inhibited by the addition of doxycycline. However, like DT40-SW cells, the remaining proteins and mRNAs of AID expressed in the cells before the addition of doxycycline can cause gene conversion after antibody selection. In contrast, the AID<sup>2</sup> system rapidly degrades AID proteins in the cells immediately (within 1 h) after the addition of auxin, allowing for the swift and almost complete abolishment of Ig gene conversion without loss of library diversity.

Despite its advantages discussed above, the AID<sup>2</sup> system still has rooms for improvement. The drawbacks of the original auxin-inducible degion system include leaky degradation and the requirement for a high dose of auxin<sup>32</sup>. Recently, improved versions of the auxin-inducible degion systems were reported by two labs using mutant TIR1s and auxin derivatives, which reduces leaky degradation and requires significantly





lower doses of ligands<sup>28,38</sup>. Application of these improved auxin-inducible degron systems would improve the performance of the AID<sup>2</sup> system. Moreover, the current construction process of AID<sup>2</sup> cells involves multiple laborious and time-consuming steps: i) heterogeneous knockout of AID, ii) homogeneous knockout of AID, iii) transfection of the TIR1 expression vector, and iv) introduction of degron-tagged AID

expression vector. Recently, methods were developed to knock-in degron-tag gene and TIR1 gene simultaneously to the gene of interest, which enables the heterologous tagging and introduction of TIR1 to in a single round of knock-in experiment<sup>28,39</sup>. With these methods, above-mentioned step iii) and iv) can be combined with i) and ii), making just two rounds of knock-in experiments to generate homogeneous knock-in

**Fig. 5 | Results of ELISA to examine the specificity of the obtained antigen-specific clones.** **a** Screening ELISA after magnetic beads selection using rIgG conjugated magnetic beads. The results for representative clones from GdAID<sup>2</sup> (left) and mAID<sup>2</sup> (right) derived libraries are shown. RIgG and ovalbumin (OA) were used as a positive and negative control antigen, respectively. **b** ELISA to examine the specificities of anti-rIgG clones. Results for representative clones obtained from GdAID<sup>2</sup> derived library (left) and mAID<sup>2</sup> (right) are shown. Apoferritin (Apo), EGFR-Fc and OA were used as negative control antigens. **c** ELISA to screen the anti-Apo clones. The results for representative clones from GdAID<sup>2</sup> (left) and mAID<sup>2</sup> (right) derived libraries are shown. Apo and OA were used as a positive and negative control antigen, respectively. **d** Screening ELISA after 3 rounds of panning from mAID<sup>2</sup> derived libraries using Apo conjugated magnetic beads, followed by limiting dilution cloning. Data for representative clones are shown. Apo and OA were used as a

positive and negative control antigen, respectively. **e** ELISA to examine the specificity of anti-Apo clone. Results for the representative clone obtained from GdAID<sup>2</sup> (left) and mAID<sup>2</sup> (right) derived library is shown. The mAID<sup>2</sup> derived clone (Anti-Apo#1(m)) is the one isolated after panning. Note that the results of the anti-Apo#4(Gd) and anti-Apo#1(m) were obtained from separate, independent experiments. RIgG, EGFR-Fc and OA were used as negative control antigens. **f** ELISA to screen the anti-EGFR clones. The results for representative clones from GdAID<sup>2</sup> (left) and mAID<sup>2</sup> (right) derived libraries are shown. EGFR-Fc and recombinant human IgG (rhIgG) were used as a positive and negative control antigen, respectively. **g** ELISA to examine the specificities of anti-EGFR clones. Results for representative clones obtained from GdAID<sup>2</sup> (left) and mAID<sup>2</sup> (right) derived libraries are shown. RIgG, Apo, OA and rhIgG were used as negative control antigens.

mutants sufficient to construct the conditional mutants applicable to AID<sup>2</sup>.

In this report, we have described the AID<sup>2</sup> system integrated to the original ADLib system that allows the antigen specific chicken IgMs. It should be noted that the AID<sup>2</sup> system can be applied to the improved versions of the ADLib systems. For example, we have previously developed the human ADLib system, which generates antigen-specific human antibodies using engineered DT40 cells<sup>19</sup>. Also in the human ADLib system, mAb libraries are diversified by gene conversion or somatic hypermutation, enabling to generate antigen-specific therapeutic mAb candidates in the form of human IgGs.

Additionally, we also have developed a DT40-based method for affinity maturation of exogenously generated mAbs<sup>40</sup>. In this method, V<sub>H</sub> and V<sub>L</sub> genes of exogenously generated mAbs, for example mAbs generated by hybridoma etc., are introduced into the Ig loci of DT40 cells. Culturing of these cells accumulates somatic hypermutation in their V regions, allowing to isolate clones with improved affinity. This knock-in affinity maturation platform (ADLib KI-Amp) technology facilitates fast-track optimization of mAbs generated by virtually any platforms. Since both the human ADLib system and the ADLib KI-Amp system utilize AID dependent Ig gene rearrangements in DT40 to generate diversified libraries, integration of the AID<sup>2</sup> system into these improved versions of the ADLib system is expected for constructing more effective and stable platforms to generate and/or optimize mAb candidates especially for medical purposes.

## Materials and methods

### Cell culture

DT40 cells were cultured at 39.5 °C in 5% CO<sub>2</sub> in IMDM containing 10% fetal bovine serum (Thermo Fisher, PA, USA, catalog no. 10270-106), 1% penicillin/streptomycin, 0.5 μM monothioglycerol and 1% chicken serum (Thermo Fisher, catalog no. 16110-082). For auxin treatment, 500 mM stock solution of auxin (Nakalai, Tokyo, Japan) in DMSO was prepared and diluted by medium.

### Construction of knock-in vector for gene conversion monitoring cell

pIN-2.6Ap, a knock-in vector for gene conversion monitoring cell was constructed as followed. First, BGH terminator was amplified by PCR using a primer set pBII-BGH-F1 and pBII-BGH-R1. As a template, synthesized BGH sequence (Azenta, MA, USA) was used. The amplified fragment was cloned into pBluescript II SK(-) digested with BamHI and PstI and, using In-Fusion system (Clontech, CA, USA) to obtain pBII-BGH plasmid. Next, puromycin resistant marker was amplified by PCR using a primer set BGH-loxRE-F1 and BGH-loxLE-R1. As a template, pH1Cp-3<sup>41</sup> was used. The amplified fragment was cloned into pBII-BGH digested with EcoRI using In-Fusion system to obtain pIN-1.0p plasmid. Next, 3' URT region of chicken Ig LC was amplified by PCR using a primer set divac-3 and divac-4. As a template, DT40 genome was used. The amplified fragment was cloned into pIN-1.0p digested with EcoRV and XhoI to obtain pIN-1.5p plasmid. Next, targeting left arm region was amplified by PCR using a primer set

pLV-1 and IgLC-2. As a template, DT40 genome was used. The amplified fragment was cloned into pIN-1.5p digested with SacI to obtain pIN-2.0Ap. Lastly, chicken Ig LC constant region was amplified by PCR using a primer set IgLJC-3 and IgLC-4. As a template, DT40 genome was used. P2A-mCherry gene was amplified by using pmCherry-C1 and cherry-2. As a template, pmCherry-C1 (Clontech) was used. These two fragments were cloned into pIN-2.0Ap digested with MluI and NotI to obtain pIN-2.6Ap.

### Construction of degron-tagged AID expression vectors

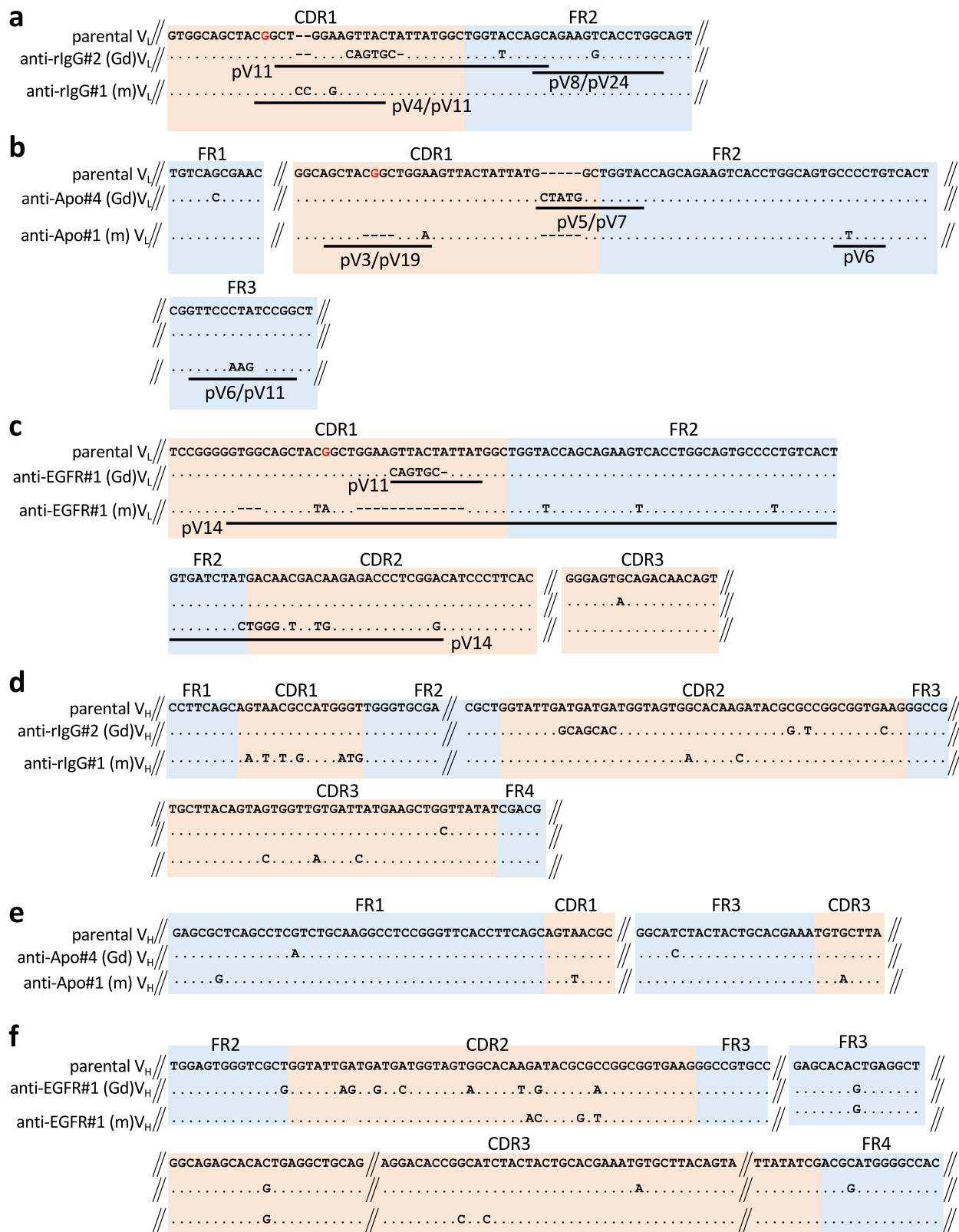
As a back born vector, p3xmAID-6xFLAG<sup>27</sup> was used and PCR to amplify inserts were carried out using KOD-FX polymerase (Toyobo, Tokyo, Japan). The sequences of the primers are described in Supplementary Table 1. As templates, cDNA of GdAID and mAID were synthesized (Azenta). For the construction of GdAID-degron expression vector and mAID-degron expression vector, inserts were amplified using primer sets 429GdAF1 and 429GdAR1, and 429mAIDF1 and 429mAIDR1, respectively. The PCR products were purified and cloned into p3xmAID-6xFLAG vector digested by NheI using In-Fusion system. For the construction of degron-GdAID expression vector and degron-mAID expression vector, inserts were amplified using primer sets 429NtermDegGdAF and 429NtermDegGdAR, and 429NtermDegmAIDF and 429NtermDegmAIDR, respectively. The PCR products were purified and cloned into p3xmAID-6xFLAG vector digested by SmaI using In-Fusion system. NLS-degron-GdAID (ΔNLS) expression vector and NLS-degron-mAID (ΔNLS) vector were constructed in two steps. First, NLS was amplified by PCR using prime set 429NtermNLSDegF and 429NtermNLSDegR, and cloned into p3xmAID-6xFLAG vector digested by SmaI using In-Fusion system. Next, cDNA of GdAID and mAID downstream of NLS were amplified by PCR using primer set I429\_DegmAID/GdA\_FLAG\_Forward and I429\_DegmAID/GdA\_FLAG\_Reverse, and cloned into NLS containing p3xmAID-6xFLAG vector digested with SmaI.

### DNA transfection

The knock-in vectors were transfected by GenePulser (Bio-Rad Laboratories, CA, USA)<sup>19</sup>. Vectors were linearized by XhoI for monitoring cell knock-in vector, NotI for AID knockout vector (kind gifts from Hiroshi Arakawa), SalI for TIR1 expression vector<sup>27</sup> and degron-tagged AID expression vectors, respectively. The electroporation was performed in the condition 600 V and 25 μF. Elimination of selection markers were performed by transfecting 6 μg of Cre recombinase expression vector (pCAG-Cre<sup>7</sup>) by Neon (Thermo Fisher) using 100 μL tips according to the manufacturer's instruction.

### Cell Sorting

Cell sorting was performed using SH800 (Sony, Tokyo, Japan). For the single-cell sorting of sIgM<sup>+</sup>/mCherry<sup>+</sup> cells for the construction of monitoring cells, 3 × 10<sup>6</sup> of cells were stained with FITC labeled anti-chicken IgM antibody (Bethyl, catalog no. A30-102F, 1:250 dilution) and single cell sorted to 96 well plate containing 200 μL of culture medium for each well. Gating strategies are described in Supplementary Fig. 8b.



**Fig. 6 | Sequence analysis of the V regions of antigen-specific clones. a** V<sub>L</sub> sequences of Anti-rlgG#2(Gd) (derived from GdAID<sup>2</sup>) and anti-rlgG#1(m) (derived from mAID<sup>2</sup>) (ELISA results are shown in Fig. 5b) are compared with the parental V<sub>L</sub> sequence (V<sub>L</sub> of parental GdAID<sup>2</sup> and mAID<sup>2</sup> cells). Dots indicate the identical nucleotides to those of parental V<sub>L</sub> whereas dashes represent deletions. Pink and blue boxes represent CDRs and FRs, respectively. Note that the red “G” in the CDR1 of parental V<sub>L</sub> represents the insertion that confer frame-shift. The horizontal bars represent gene conversion tracts and corresponding pseudogenes are described besides (pV11 etc.). **b** V<sub>L</sub> sequences of Anti-Apo#4(Gd) and Anti-Apo#1(m) (ELISA

result is shown in Fig. 5e) are compared with the parental V<sub>L</sub> sequence. **c** V<sub>L</sub> sequences of Anti-EGFR#1(Gd) and anti-EGFR#2(m) (ELISA results are shown in Fig. 5g) are compared with the parental V<sub>L</sub> sequence. **d** V<sub>H</sub> sequences of Anti-rlgG#2(Gd) and anti-rlgG#2(m) are compared with the parental V<sub>H</sub> sequence. **e** V<sub>H</sub> sequence of Anti-Apo#4(Gd) and Apo#1(m) are compared with the parental V<sub>H</sub> sequence. **f** V<sub>H</sub> sequences of Anti-EGFR#1(Gd) and anti-EGFR#2(m) are compared with the parental V<sub>H</sub> sequence. All the sequences have been registered in GenBank, with the accession numbers PQ885560 to PQ885571.

## Genotyping PCR

Knock-in was examined by PCR using KOD-FX polymerase (Toyobo). Primer sets are shown in Supplementary Fig. 2a and Supplementary Fig. 3a. The sequences of the primers are described in Supplementary Table 1.

## Flow Cytometry

To examine the expression level of chicken IgM, cells ( $1 \times 10^6$ ) were washed twice with FACS buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA) and stained with FITC labeled anti-chicken IgM antibody (Bethyl, catalog no. A30-102F, 1:250 dilution) for 15 m. Cells were washed twice by FACS buffer and analyzed by NovoCyte (Agilent, CA, USA) or SH800. For the detection of chicken Ig $\lambda$ , unlabeled mouse anti-chicken Ig $\lambda$  monoclonal antibody (Southern Biotech, AL, USA, catalog no. 8340-01) was labeled with biotin using Biotin Labeling Kit-NH<sub>2</sub> (Dojindo, Kumamoto, Japan, catalog no. LK03) and used identically to anti-chicken IgM antibody except that FITC-labeled streptavidin (BioLegend, 1:1000 dilution) was added. Gating strategies are described in Supplementary Fig. 8a, c.

## Immunoblotting

$10^6$  cells were washed with 500  $\mu$ L of PBS (containing auxin if necessary), lysed with 100  $\mu$ L of RIPA buffer containing 1  $\times$  cComplete (Roche, Basel, Switzerland) and incubated at 4 °C for 15 m followed by the centrifugation (20,400  $\times$ g, 10 m). The supernatants were collected and diluted 1:1 in 2  $\times$  Laemmli loading buffer, denatured 5 m at 95 °C separated by SDS-PAGE, and transferred onto nitrocellulose membranes (Hybond-ECL, GE-Healthcare, CA, USA). The blots were blocked in PBST (1 $\times$  PBS, 0.05% Tween) with 5% skim milk, incubated with appropriate antibodies [1:2000 mouse anti-chicken Ig $\lambda$  monoclonal antibody (Southern Biotech, catalog no. 8340-01), 1:5000 rabbit anti-mCherry polyclonal antibody (GeneTex, LA, USA, catalog no. GTX128508), 1:1000 rabbit anti-Myc-tag polyclonal antibody (MBL, catalog no. 562), 1:2000 mouse anti-mini-AID-tag monoclonal antibody (anti-degron antibody) (MBL, Tokyo, Japan, catalog no. M214-3) and 1:2000 rabbit anti- $\beta$ -actin polyclonal antibody (MBL, catalog no. PM053)] and washed TBST five times. The membrane was further incubated with secondary antibody [1:2000 HRP-labeled anti-mouse IgG antibody (Cell Signaling, MA, USA, catalog no. 7076) and 1:2000 HRP-labeled anti-rabbit IgG antibody (Cell Signaling, catalog no. 7074)] and washed with PBST for five times. Signals were detected by Image Quant LAS4000 mini (GE Healthcare) using the ECL prime western blotting reagents (GE Healthcare).

## Selection of antigen-specific clones

As antigen proteins, commercially available rIgG (SIGMA, MO, USA), Apoferritin (SIGMA), human EGFR-Fc (R&D systems, MN, USA), ovalbumin (SIGMA) and rhIgG (R&D systems) were purchased. For magnetic beads selection, antigens were conjugated with Dynabeads M-280 Tosyl-activated (ThermoFisher) according to the manufacturer's instruction. Then antigen-coated magnetic beads and AID<sup>2</sup> based libraries ( $1 \times 10^8$  cells for each) were mixed in 1 mL of selection buffer (1% BSA in PBS) and incubated for 30 m at 4 °C, gently rotating. After washing three times with selection buffer using a magnetic stand (Dyna MPC-S) for 2 m, the recovered cells were diluted with fresh medium, dispensed into 96 well plates and incubated for 1 week in a CO<sub>2</sub> incubator at 39.5 °C. DT40 cells expressing the antigen-specific antibodies were screened by ELISA.

## ELISA

Maxisorp immunoplates (Nunc) were incubated overnight at 4 °C with the antigen diluted at 3  $\mu$ g/mL in PBS. After blocking with 1% BSA in PBS for 30 m at room temperature and washing three times with PBST, culture supernatants were added to the plates and incubated for 1 h at room temperature. After washing five times with PBST, HRP-labeled anti-chicken IgM (Bethyl, catalog no. A30-103P, 1:10,000 dilution), solution was added to the plates and incubated for 1 h at room temperature. After washing the plates five times with PBST, 3,3',5,5'-tetramethyl- benzidine (TMB) (Dako Cytomation, Glostrup, Denmark) was added, and the reaction was stopped

with 1 N sulfuric acid. The optical density at 450 nm was measured with a microplate reader Model 680 (Bio-Rad). All the raw data are available in Supplementary Data.

## Competition ELISA

Antigen-specific culture supernatants were serially diluted with fresh culture medium to achieve an optimal density of approximately OD = 0.5 in a standard solid phase ELISA as described above. For competition assays, anti-apoferritin culture supernatants were further incubated with serial 1/4 dilutions of apoferritin, starting at a maximum concentration of 2  $\mu$ M. For anti-rIgG culture supernatants, we used serial 1/3 dilutions of rIgG, starting at a maximum concentration of 10  $\mu$ M. These mixtures were then subjected to solid phase ELISA to measure competitive binding. All the raw data are available in Supplementary Data.

## Sequence analysis of V regions

The genomes of DT40 cells were extracted by FavorPrep Blood Genomic DNA Extraction Mini Kit (FAVORGEN, Taipei Taiwan) and V<sub>L</sub> and V<sub>H</sub> were amplified by PCR using primer sets VL\_seq\_F1 and VL\_seq\_R1, and VH\_seq\_F1 and VH\_seq\_R1, respectively. The sequences of the primers are described in Supplementary Table 1. The amplified PCR products were electrophoresed and DNA was recovered from the corresponding bands using FavorPrep Gel Purification Mini Kit (FAVORGEN). The sequences of the recovered DNA were analyzed using forward and reverse primer used for PCR amplification.

The analysis of V<sub>H</sub> sequences of auxin-treated cells (Fig. 4c) was conducted as follows: mCherry<sup>+</sup> subclones were single cell sorted from mAd1-4 using SH800 onto 96 well plates containing medium with or without 1 mM auxin for 6 days. From each plate, six subclones were selected. These clones were further cultured for 8 days with or without 1 mM auxin. Genomic DNA was then extracted from the cells. V<sub>H</sub> were amplified by PCR using the primer set pBS2SKM-HV-1 and HV-2-pBS2SKM, and the products were cloned into pBlueScriptII SK(-), previously digested with EcoRI and NotI, using In-Fusion system. Transformation was carried out in DH5 $\alpha$ . Colony PCR was performed using M13 forward and reverse primers. The PCR products were directly sequenced by sanger sequencing using HV-1 and HV-2 as sequence primers.

## Statistics and reproducibility

All the numerical raw data are available in Supplementary Data. Statistical calculations were performed with GraphPad Prism 10 and are described in the relevant figure legends. Differences were compared using a two-tailed *t*-test. The statistically significant difference between the two groups was defined as  $P < 0.05$  \*. The sample sizes for the fluctuation assays were 24, except for mp232- $\Delta$ AID in Fig. 3b, which was 23. IC50 values were calculated using the dose-response inhibition model in GraphPad Prism 10. All data sets correspond to at least three biological replicates, except for the screening ELISA shown in Fig. 5a, c, d, and f, which was conducted as a single-round experiment. The reproducibility of these results is further confirmed by additional ELISA tests examining antigen-specificities, as depicted in Fig. 5b, e, g.

## Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## Data availability

All data and materials in this paper will be available from the corresponding author upon reasonable request. The numerical source data are available in Supplementary Data. Uncropped and unedited blot/gel images are shown in Supplementary Figs. 9–11. The sequence data are deposited to GenBank with accession numbers for the sequences are as follows: anti-Apo#4\_(Gd) VH is PQ885560, anti-Apo#1\_(m)VH is PQ885561, anti-EGFR#1\_(Gd) VH is PQ885562, anti-EGFR#1\_(m)VH is PQ885563, anti-rIgG#2\_(Gd) VH is PQ885564, anti-rIgG#1\_(m)VH is PQ885565, anti-Apo#4\_(Gd)VL



is PQ885566, anti-Apo#1\_(m)VL is PQ885567, anti-EGFR#1\_(Gd)VL is PQ885568, anti-EGFR#1\_(m)VL is PQ885569, anti-rIgG#2\_(Gd)VL is PQ885570, and anti-rIgG#1\_(m)VL is PQ885571.

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## References

- Köhler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497 (1975).
- Smith, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Sci. (80-)* **228**, 1315–1317 (1985).
- Boder, E. T. & Wittrup, K. D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**, 553–557 (1997).
- Hanes, J. & Plückthun, A. In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA* **94**, 4937–4942 (1997).
- Seo, H. et al. Rapid generation of specific antibodies by enhanced homologous recombination. *Nat. Biotechnol.* **23**, 731–735 (2005).
- Seo, H. et al. An ex vivo method for rapid generation of monoclonal antibodies (ADLib system). *Nat. Protoc.* **1**, 1502–1506 (2006).
- Hashimoto, K., Kurosawa, K., Murayama, A., Seo, H. & Ohta, K. B Cell-Based Seamless Engineering of Antibody Fc Domains. *PLoS One* **11**, 1–22 (2016).
- Hashimoto, K., Kurosawa, K., Seo, H. & Ohta, K. Rapid Chimerization of Antibodies. *Methods Mol. Biol.* **1904**, 307–317 (2019).
- Reynaud, C. A., Anquez, V., Grimal, H. & Weill, J. C. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell* **48**, 379–388 (1987).
- Reynaud, C. A., Dahan, A., Anquez, V. & Weill, J. C. Somatic hyperconversion diversifies the single Vh gene of the chicken with a high incidence in the D region. *Cell* **59**, 171–183 (1989).
- Abe, T., Branzel, D. & Hirota, K. DNA damage tolerance mechanisms revealed from the analysis of immunoglobulin v gene diversification in avian DT40 cells. *Genes (Basel)* **9**, 614 (2018).
- Buerstedde, J. M. & Takeda, S. Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell* **67**, 179–188 (1991).
- Buerstedde, J. M. et al. Light chain gene conversion continues at high rate in an ALV-induced cell line. *EMBO J.* **9**, 921–927 (1990).
- Seo, H., Yamada, T., Hashimoto, S., Lin, W. & Ohta, K. Modulation of Immunoglobulin Gene Conversion in Chicken DT40 by Enhancing Histone Acetylation, and its Application to Antibody Engineering Modulation of Immunoglobulin Gene Conversion in Chicken DT40 by Enhancing Histone Acetylation, and its. *Biotechnol. Genet. Eng. Rev.* **24**, 179–194 (2007).
- Guy, A. T. et al. Glycerophospholipid regulation of modality-specific sensory axon guidance in the spinal cord. *Sci. (80-)* **349**, 974–977 (2015).
- Yamashita, N. et al. Anti-semaphorin 3A neutralization monoclonal antibody prevents sepsis development in lipopolysaccharide-treated mice. *Int. Immunol.* **27**, 459–466 (2015).
- Kanemaru, H., Yamada, Y., Ohazama, A., Maeda, T. & Seo, K. Semaphorin 3A Inhibits Nerve Regeneration During Early Stage after Inferior Alveolar Nerve Transection. *Sci. Rep.* **9**, 1–7 (2019).
- Omi, K. et al. Noncompetitive immunoassay detection system for haptens on the basis of antimetatype antibodies. *Clin. Chem.* **61**, 627–635 (2015).
- Seo, H. et al. Streamlined human antibody generation and optimization by exploiting designed immunoglobulin loci in a B cell line. *Cell. Mol. Immunol.* **18**, 1545–1561 (2020).
- Muramatsu, M. et al. Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (AID), a Potential RNA Editing Enzyme. *Cell* **102**, 553–563 (2000).
- Arakawa, H., Hauschild, J. & Buerstedde, J.-M. Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. *Sci. (80-)* **295**, 1301–1306 (2002).
- Harris, R. S., Sale, J. E., Petersen-Mahrt, S. K. & Neuberger, M. S. AID is essential for immunoglobulin V gene conversion in a cultured B cell line. *Curr. Biol.* **12**, 435–438 (2002).
- Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat. Methods* **6**, 917–922 (2009).
- Samejima, K. et al. Auxin-induced rapid degradation of inhibitor of caspase-activated DNase (ICAD) induces apoptotic DNA fragmentation, caspase activation, and cell death: A cell suicide module. *J. Biol. Chem.* **289**, 31617–31623 (2014).
- Daly, O. M. et al. CEP164-null cells generated by genome editing show a ciliation defect with intact DNA repair capacity. *J. Cell Sci.* **129**, 1769–1774 (2016).
- Bulley, S. J. et al. In B cells, phosphatidylinositol 5-phosphate 4-kinase- $\alpha$  synthesizes PI(4,5)P2 to impact mTORC2 and Akt signaling. *Proc. Natl. Acad. Sci. USA* **113**, 10571–10576 (2016).
- Kawasumi, R. et al. ESCO1/2's roles in chromosome structure and interphase chromatin organization. *Genes Dev.* **31**, 2136–2150 (2017).
- Nishimura, K. et al. A super-sensitive auxin-inducible degron system with an engineered auxin-TIR1 pair. *Nucleic Acids Res.* **48**, E108 (2020).
- Nakahara, M. et al. Genetic evidence for single-strand lesions initiating Nbs1-dependent homologous recombination in diversification of Ig V in chicken B lymphocytes. *PLoS Genet.* **5**, e1000356 (2009).
- Kohzaki, M. et al. DNA polymerases  $\nu$  and  $\theta$  are required for efficient immunoglobulin V gene diversification in chicken. *J. Cell Biol.* **189**, 1117–1127 (2010).
- Kim, J. H. et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* **6**, 1–8 (2011).
- Yesbolatova, A. et al. The auxin-inducible degron 2 technology provides sharp degradation control in yeast, mammalian cells, and mice. *Nat. Commun.* **11**, 5701 (2020).
- Seo, H., Hirota, K. & Ohta, K. Molecular mechanisms of avian immunoglobulin gene diversification and prospect for industrial applications. *Front. Immunol.* **15**, 1–8 (2024).
- Lin, W., Kurosawa, K., Murayama, A., Kagaya, E. & Ohta, K. B-cell display-based one-step method to generate chimeric human IgG monoclonal antibodies. *Nucleic Acids Res.* **39**, e14–e14 (2011).
- Kanayama, N., Todo, K., Reth, M. & Ohmori, H. Reversible switching of immunoglobulin hypermutation machinery in a chicken B cell line. *Biochem. Biophys. Res. Commun.* **327**, 70–75 (2005).
- Todo, K., Miyake, K., Magari, M., Kanayama, N. & Ohmori, H. Novel in vitro screening system for monoclonal antibodies using hypermutating chicken B cell library. *J. Biosci. Bioeng.* **102**, 478–481 (2006).
- Wang, B., Wang, F., Huang, H. & Zhao, Z. A Novel DT40 Antibody Library for the Generation of Monoclonal Antibodies. *Viro. Sin.* **34**, 641–647 (2019).
- Yesbolatova, A., Natsume, T., Hayashi, K. I. & Kanemaki, M. T. Generation of conditional auxin-inducible degron (AID) cells and tight control of degron-fused proteins using the degradation inhibitor auxinole. *Methods* **164–165**, 73–80 (2019).
- Yesbolatova, A., Saito, Y. & Kanemaki, M. T. Constructing auxin-inducible degron mutants using an all-in-one vector. *Pharmaceuticals* **13**, 1–8 (2020).
- Masuda, H. et al. Fast-tracking antibody maturation using a B cell-based display system. *MAbs* **14**, 2122275 (2022).
- Kurosawa, K., Lin, W. & Ohta, K. Distinct roles of HDAC1 and HDAC2 in transcription and recombination at the immunoglobulin loci in the chicken B cell line DT40. *J. Biochem.* **148**, 201–207 (2010).



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## Author contributions

A.M. and S.M. contributed to experiments and data analysis. T.A. contributed to research design and critically reviewed the manuscript. M.T.K. critically reviewed the manuscript. K.K. conceived the project and critically reviewed the manuscript. K.H. contributed to research design and critically reviewed the manuscript. K.O. supervised the project and critically reviewed the manuscript. H.S. conceived the project, contributed to research design, data analysis, and drafted the manuscript.

## Competing interests

This work was supported by Chiome Bioscience Inc. (Japan). K.K. is an employee of Chiome Bioscience Inc. H.S. is a former employee of Chiome Bioscience Inc. K.O. is a former board member of Chiome Bioscience Inc. K.K., H.S. and K.O. are stockholders of Chiome Bioscience Inc. K.K. is a stock option holder of Chiome Bioscience.

## Additional information

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