1 Scarless Enriched selection of Genome edited Human Pluripotent Stem Cells

2 Using Induced Drug Resistance

3 Running title: Efficient scarless selection of edited hPSCs

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24 **Key words:** Genome editing, CRISPR, Knockout, YM155, *SLC35F2*, Human

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26

1 **Summary**

2 An efficient gene editing technique for use in human pluripotent stem cells
3 (hPSCs) would have great potential value in regenerative medicine, as well as in drug
4 discovery based on isogenic human disease models. However, the extremely low
5 efficiency of gene editing in hPSCs is a major technical hurdle that remains to be
6 resolved. Previously, we demonstrated that YM155, a survivin inhibitor developed as
7 an anti-cancer drug, induces highly selective cell death in undifferentiated hPSCs. In
8 this study, we demonstrated that the high cytotoxicity of YM155 in hPSCs, which is
9 mediated by selective cellular uptake of the drug, is due to high expression of
10 *SLC35F2* in these cells. Consistent with this, knockout of *SLC35F2* with CRISPR-
11 Cas9 or depletion with siRNAs made hPSCs highly resistant to YM155. Simultaneous
12 gene editing of a gene of interest and transient knockdown of *SLC35F2* following
13 YM155 treatment enabled genome-edited hPSCs to survive because YM155
14 resistance was temporarily induced, thereby achieving enriched selection of genome-
15 edited clonal populations. This precise and efficient genome editing approach took as
16 little as 3 weeks without cell sorting or introduction of additional genes.

17

18 **Keywords:** Gene editing, CRISPR/Cas9, CCR5, SLC35F2, YM155, human
19 pluripotent stem cells, disease modeling

20

1 **Introduction**

2 Along with recent advances in human pluripotent stem cells (hPSCs) (Avior et
3 al., 2016; Shi et al., 2017), genome engineering in hPSCs has shown great potential
4 for gene correction in the context of regenerative medicine (Hockemeyer and Jaenisch,
5 2016). The use of hPSCs derived from patients with genetic disorders enables a
6 constant supply of certain types of cells that mimic the pathology of interest (i.e.,
7 disease modeling), and can thus make valuable contributions to both drug screening
8 and basic studies (Engle and Puppala, 2013; Hockemeyer and Jaenisch, 2016).
9 However, patient-derived induced PSCs (iPSCs) require large numbers of case and
10 control iPSCs to minimize the effects of variability among unrelated hPSC lines. By
11 contrast, genome-edited hPSCs provide isogenic pairs of control and disease model
12 cells, enabling rigorous comparisons (Hendriks et al., 2016; Merkle and Eggen, 2013;
13 Musunuru, 2013).

14 Despite the great potential of genome engineering in hPSCs, the extremely
15 low efficiency of this procedure, along with the time-consuming and laborious
16 procedures required for clonal selection, remains a critical obstacle to a wide range of
17 applications, such as pooled sgRNA screening, which can be performed in mouse
18 ESCs (Koike-Yusa et al., 2014). Another factor contributing to this technical barrier is
19 the fact that Cas9 activity in hPSCs is low (Mali et al., 2013); moreover, the cells
20 undergo massive p53-dependent cell death in response to DNA damage by effect of
21 Cas9 (Ihry et al., 2018). Multiple strategies have been developed to address the low
22 efficiency of genome editing in hPSCs (as well as in other somatic cell models):
23 inducible Cas9 systems (Cao et al., 2016; Gonzalez et al., 2014), puromycin selection
24 (Steyer et al., 2018), self-replicating episomal vectors (Xie et al., 2017), and so on.
25 Alternatively, enriched selection of a minority of the target-edited clones has been

1 attempted by a ‘co-targeting’ approach that enables any target-edited clone to survive
 2 through acquired resistance induced by knockout of a gene responsible for drug
 3 sensitivity (Agudelo et al., 2017; Liao et al., 2015). However, it would be preferable
 4 to achieve ‘scarless enrichment’ of target-edited clones without creating permanent
 5 ‘scars’ or introducing foreign genes (e.g., manipulation of a gene responsible for
 6 induction of drug resistance), in order to minimize undesirable bias from the
 7 additional manipulation.

8 Teratoma formation resulting from unintended transplantation of
 9 undifferentiated hPSCs is a serious risk of hPSC-based cell therapy (Lee et al., 2013a).
 10 To address this danger, several approaches have been developed for selective removal
 11 of residual hPSCs (Jeong and Cha, 2017). For example, we previously reported that
 12 YM155, a survivin inhibitor developed as an anti-cancer drug, is selectively cytotoxic
 13 against undifferentiated hPSCs and inhibits teratoma formation (Lee et al., 2013b),
 14 and this finding has been reproduced in several independent studies (Bedel et al.,
 15 2017; Kang et al., 2017; Kim et al., 2017). At that time, however, the molecular
 16 mechanism underlying the high susceptibility of hPSCs to YM155 had not been fully
 17 elucidated.

18 In this study, we found that selective cellular uptake of YM155, which is
 19 responsible for the selective cell death of undifferentiated hPSCs, is due to high
 20 expression of *SLC35F2*, which encodes a solute carrier membrane transporter.
 21 Introduction of gene editing single guide RNA (sgRNA) targeting a gene of interest in
 22 parallel with transient siRNA-mediated knockdown of *SLC35F2* efficiently achieved
 23 enriched selection of genome-edited hPSCs via induced YM155 resistance. Moreover,
 24 this scarless approach to highly efficient enriched selection did not require laborious
 25 clonal selection, and thus allowed a single clone to be obtained in as little as 3 weeks.

26

1 **Results**

2 **High expression of *SLC35F2* mediates intracellular uptake of YM155 in hPSCs**

3 Selective induction of cell death in hPSCs by YM155, which decreases
4 teratoma formation (Bedel et al., 2017; Kang et al., 2017; Kim et al., 2017; Lee et al.,
5 2013b), is associated with the DNA damage response (Lee et al., 2013b). Cytotoxicity
6 of YM155 cannot be fully prevented by suppression of *BIRC5* (which encodes
7 survivin) (Sim et al., 2017). Hence, we sought to identify the mechanism underlying
8 selective cell death of hPSCs. To this end, we took advantage of bulk cancer cell line
9 gene expression data and drug response data from the Cancer Cell Line Encyclopedia
10 (CCLE) and Cancer Therapeutics Response Portal (CTRP), respectively. First, using
11 data from gene expression omnibus (GEO) (Table. S1), we identified a hPSC
12 signature, i.e., a set of genes that are differentially expressed in hPSCs relative to their
13 differentiated counterparts. Using the hPSC signature, we calculated hPSC scores
14 (Materials and Methods in detail) for 666 human cancer cells by performing single-
15 sample Kolmogorov–Smirnov (KS) enrichment analysis. By correlating the hPSC
16 scores with the sensitivities of each cell line to each of 543 compounds, we identified
17 YM155 as the most effective drug in cells with high hPSC scores (Fig. 1B); this
18 implies that YM155 should be highly specific for hPSCs, as demonstrated previously
19 (Bedel et al., 2017; Kang et al., 2017; Kim et al., 2017; Lee et al., 2013b). Based on
20 correlation of drug and gene expression signature (Fig. 1A), genes whose expression
21 was highly correlated with sensitivity to YM155 were identified as described
22 previously (Rees et al., 2016). *SLC35F2*, a membrane solute carrier, was the transcript
23 most correlated with YM155 sensitivity (Fig. 1B, left panel), suggesting that cellular
24 import of YM155 by *SLC35F2* is responsible for the highly selective cytotoxicity of
25 the drug in hPSCs (Lee et al., 2013b); this is consistent with previous reports in

cancer models (Nyquist et al., 2017; Radic-Sarikas et al., 2017; Winter et al., 2014). Using a database of gene expression profiles (<http://nextbio.com>) (Kupersmidt et al., 2010), we compared relative *SLC35F2* expression between 24 human embryonic stem cells (hESCs) and various cancer cell lines; PC-3, a human prostate cancer cell line, was used as a positive control (P.C.) for high expression of *SLC35F2* (Winter et al., 2014). As shown in Figure 1C, all hESCs (including H9, mainly used in this study) expressed *SLC35F2* at higher levels than the other cancer cell lines, consistent with a previous study that identified hESC surface marker proteins (Kolle et al., 2009). On the other hand, mesenchymal stem cells derived from hESCs (hESC-MSCs) and human dermal fibroblasts (hDFs), which are both highly resistant to YM155 treatment (Kim et al., 2017; Lee et al., 2013b), expressed lower levels of *SLC35F2* than two independent hESC lines (H9 and hCHA3) and induced pluripotent stem cells (iPSCs: SES8) (Lee et al., 2013b) (Figs. 1D and E). *SLC35F2* is the membrane transporter responsible for uptake of YM155 into cells, resulting in DNA damage (Winter et al., 2014); consistent with this, marked DNA damage was detected solely in hESCs, but not in hESC-MSCs, following YM155 treatment (Figs. 1F and G). The higher intracellular level of YM155 in hESCs relative to hDFs (Fig. 1H) clearly indicates that high *SLC35F2* expression in hPSCs results in selective cytotoxicity of this drug in hPSCs.

SLC35F2 is responsible for YM155-induced selective cell death in hPSCs

To explore the connection between the selective cytotoxicity of YM155 in hPSCs (EC_{50} : 10 nM, Fig. S1B) and the high expression of *SLC35F2* in these cells (Figs. 1D, E, and S1A), we knocked out *SLC35F2* in hESCs using CRISPR/Cas9 by targeting exon 7, as previously described (Winter et al., 2014) (Fig. 2A). After

1 introducing a single guide RNA (sgRNA) against *SLC35F2* along with Cas9, we
 2 treated the cells with YM155 to ablate wild-type hESCs that had not been edited.
 3 Following YM155 treatment, a few colonies survived, whereas most cells underwent
 4 cell death (Fig. 2B, red arrow). One of the surviving colonies (Fig. 2B, YM155-
 5 resistant clone: YM155R) was selected and maintained. Clone YM155R was highly
 6 resistant to further YM155 treatment (Fig. 2B, right panel). Furthermore, after
 7 introduction of the sgRNA against *SLC35F2* and Cas9, insertion/deletion (indel)
 8 frequency was increased in a concentration-dependent manner (Fig. 2C), and in the
 9 presence of 100 nM YM155, the surviving clones were nearly 100% genome-edited
 10 hESCs (100% indel) (Fig. 2D), suggesting that the *SLC35F2* knockout (*SLC35F2*
 11 KO) hESC population was enriched in a dose-dependent manner, thereby efficiently
 12 eliminating false positives (i.e., hESCs that survived with wild-type *SLC35F2*). A
 13 single clone from YM155R maintained under YM155 treatment turned out to be a
 14 homozygous bi-allelic *SLC35F2* KO (Fig. 2E). This clone (*SLC35F2* KO hESCs:
 15 KO#1) was highly resistant to YM155-induced cell death (Fig. 2F and Movie S1).
 16 However, like control hESCs, it underwent dramatic cell death in response to
 17 bleomycin (BLM), which is imported by SLC22A16, another member of the solute
 18 carrier family (Aouida et al., 2010) (Fig. S1C). We concluded that the acquired
 19 resistance of *SLC35F2* KO hESCs to YM155 was the result of low intracellular levels
 20 of YM155 due to failure of drug uptake (Fig. 2H) and subsequent fewer DNA damage
 21 response in the absence of *SLC35F2* (Fig. 2G).

22 To characterize the *SLC35F2* KO hESCs, we monitored the expression levels
 23 of typical pluripotency markers such as *NANOG*, *SOX2*, and *POU5F1*. Consistent
 24 with the absence of alteration of typical pluripotency markers (Fig. 3A), protein levels
 25 of SOX2, OCT4, and LIN28A (Figs. 3B and S1D), as well as alkaline phosphatase

1 activity (Fig. 3C), were not significantly altered in *SLC35F2* KO hESCs. Consistent
2 with this, self-renewal of *SLC35F2* KO hESCs was also maintained, as reflected by
3 their growth rate (Figs. 3D and S1E), fluorescence-based competition assay with WT
4 control (Fig. 3E) (Cha et al., 2017), and *in vitro* and *in vivo* differentiation potentials
5 (Figs. 2F and G). In support of these findings, comparison of transcriptome data from
6 WT and KO hESCs revealed a linear relationship in the expression levels of
7 pluripotency genes (Korkola et al., 2006) (red circles) (Fig. 3H).

8

9 **YM155-mediated enriched selection**

10 The observation that enriched selection of *SLC35F2* KO hESCs could be
11 achieved by YM155 treatment led us to predict that induced resistance to YM155
12 could be useful for isolating genome-edited hPSCs if *SLC35F2* were co-targeted with
13 the gene of interest (GOI) (Fig. 4A). For a proof-of-concept study, we took advantage
14 of HEK293T cells, which express relatively high levels of *SLC35F2* (Fig. S2A),
15 leading to dose-dependent cell death following YM155 treatment (10-fold higher
16 EC₅₀ than for hESCs, Fig. S2B). Simple introduction of sgRNA for *SLC35F2* along
17 with Cas9, followed by YM155 treatment, led to establishment of a genome-edited
18 population (Fig. 4B) at up to 88.4% efficiency (Fig. 4C). Like hESCs, *SLC35F2* KO
19 HEK293T cells were highly resistant to YM155, but not doxorubicin (Fig. S2C and
20 D), due to failure of cellular uptake of YM155 (Fig. S2E).

21 Next, we attempted co-targeting of *SLC35F2* along with the *CCR5*. In these
22 experiments, we took advantage of the *CCR5* surrogate reporter system (Ramakrishna
23 et al., 2014) to monitor the efficiency of *CCR5* targeting. In this system, the
24 monomeric red fluorescent protein (mRFP) is expressed constitutively, whereas
25 expression of green fluorescent protein (GFP) is initiated only after *CCR5* is targeted

by Cas9 expression (Fig. 4D). Thus, the proportion of GFP-positive cells enables live monitoring of CRISPR/Cas9 targeting efficiency. After co-targeting *SLC35F2* and *CCR5*, followed by YM155 treatment, we observed a significant increase in the GFP-positive population (Figs. 4E and F). Considering that the surrogate reporter system reveals only one-third of the genome-edited population (Ramakrishna et al., 2014), the ~25% GFP-positive population following YM155 selection implied a genome-edited population as high as 80% (Fig. 4F). It is also noteworthy that YM155 treatment eliminated most mRFP-expressing cells (red box), thereby selectively enriching for GFP-positive cells (blue box) (Fig. S2F, Movies S1 and S2). To confirm enrichment of *CCR5* KO by co-targeting of *SLC35F2* followed by YM155 treatment, we performed the T7 endonuclease 1 (T7E1) assay for *CCR5*; the results revealed a distinct increase in KO efficiency (Fig. 4G).

YM155-based enriched selection of genome-edited hESCs

After testing this approach (YM155-based Enriched Selection of CRISPR Co-target; hereafter, ‘YES-approach’) in HEK293T cells (Fig. 4), we applied it to hPSCs, which are more sensitive to YM155 (Figs. 1 and 2). For proof-of-concept of the YES-approach, we attempted to produce *CCR5*-targeted hESCs. We chose this gene for the following reasons: (i) disruption of *CCR5* (a genomic safe harbor (Sadelain et al., 2011)) has little effect on the pluripotency of hPSCs (Kang et al., 2015); and (ii) the cells would have potential future applications in HIV-1 research, e.g., production of *CCR5*-depleted CD4⁺ T cells (Holt et al., 2010; Hutter et al., 2009; Lai, 2012). In these experiments, we used two different ratios of sgRNAs targeting *CCR5* and *SLC35F2* to minimize the selection of false-positive clones (e.g., *CCR5*WT/*SLC35F2*KO) that could still survive under YM155 treatment. Through the

1 YES-approach with sgRNA targeting *CCR5* (C) and *SLC35F2* (S), seven (for
2 C:S=1:1 ratio) or four (C:S=2:1) colonies survived and were isolated. Among them,
3 three clones and one clone, respectively, were *CCR5*-targeted hESCs (over 25%
4 efficiency) (Fig. 5A). Accordingly, a single clone after the selection was shown as
5 85.5% indel occurred, implying that the surviving clones were successfully genome-
6 edited in the desired manner (Fig. 5B).

7 Although overall gene expression and expression of pluripotency genes were
8 not altered in *SLC35F2* KO hESCs (Fig. 3H), we could not rule out the effect of
9 permanent KO of *SLC35F2* in hESCs. Therefore, we closely examined the genes
10 whose expression was altered in *SLC35F2* KO cells. Although we detected only
11 minor differences between wild-type (NC#1 and NC#2) and KO cells (KO#1 and
12 KO#2) in both overall transcription and the hPSC signature, expression of genes
13 involved in ‘cellular transition metal ion homeostasis,’ which are associated with the
14 original function of *SLC35F2* as a membrane transporter, was altered significantly
15 (Fig. 5C and Table. S2).

16

17 **Scarless YES-approach for establishment of *CCR5*-targeted hESCs**

18 Although the YES-approach was effective for establishing GOI-targeted
19 hESCs, permanent KO of *SLC35F2* to induce YM155 resistance would be
20 problematic from the standpoint of isogenic disease modeling because the effects of
21 *SLC35F2* on mineral or ion homeostasis (Fig. 5C and Table S2) could not be ruled
22 out. To address this complication, transient induced YM155 resistance mediated by
23 depletion of *SLC35F2* was achieved by introduction of a siRNA against *SLC35F2*
24 along with the sgRNA targeting the GOI (in this case, *CCR5*) (Fig. 6A). As predicted,
25 depletion of *SLC35F2* had fully recovered 5 days after siRNA transfection (Fig. 6B).

1 Induced YM155 resistance disappeared at the same time (Fig. 6C, right panel),
 2 allowing for enriched selection at 2 days after siRNA transfection (Fig. 6C, left panel).
 3 These observations suggest that induced YM155 resistance mediated by siRNA
 4 against *SLC35F2* occurred transiently, with no permanent scar.

5 We then applied this scarless YES-approach to *CCR5* gene editing in hESCs.
 6 As shown in Figure 6D, *CCR5* target efficiency was improved in a YM155 dose-
 7 dependent manner. Notably in this regard, the indel percentage in samples with the
 8 YES-approach reached 72.2% even without laborious isolation of numerous single
 9 colonies (Figs. S3A-D). Accordingly, the mutation patterns of the genome-edited
 10 population were simplified by treatment with 20 nM YM155, indicating that few
 11 colonies survived the treatment (Figs. 6E and S3E). One of the clones obtained from
 12 the YES-approach was correctly targeted to *CCR5* (Figs. 6F and S3F). Also, the
 13 *CCR5* targeted clone maintained pluripotency as determined by alkaline phosphatase
 14 activity and expression level of pluripotency markers (Fig. S3G). Along with recovery
 15 of *SLC35F2* expression (Fig. S3G), the cytotoxicity of YM155 was fully restored
 16 (Figs. 6G and S3H), suggesting the functional recovery of *SLC35F2*.

17

18

1 Discussion

2 In order to avoid the laborious procedures made necessary by the extremely
3 low efficiency of genome editing using current techniques, a variety of strategies have
4 been developed to improve editing efficiency not only in hPSCs (Gonzalez et al.,
5 2014; Mitzelfelt et al., 2017; Steyer et al., 2018), but also in somatic cell models
6 (Agudelo et al., 2017; Cao et al., 2016; Liao et al., 2015). Among them, enriched
7 selection approaches based on inducing drug resistance by targeting the causative
8 gene responsible for drug sensitivity (Agudelo et al., 2017; Liao et al., 2015) would
9 be advantageous because selection of desirable clones results from altered phenotype
10 (e.g., induced drug resistance) caused by effect of gene editing unlike puromycin
11 selection, corresponding to merely Cas9 expression (Steyer et al., 2018). In these
12 procedures, however, induced drug resistance was achieved by producing a
13 permanent scar in the causative genes (Agudelo et al., 2017; Liao et al., 2015),
14 potentially resulting in unexpected bias in hPSCs-based disease models.

15 Here, we demonstrated that selective induction of cell death in hPSCs by
16 YM155 (Lee et al., 2013b) results from selective cellular uptake of the drug due to
17 high expression of *SLC35F2* (Figs. 1 and S1A). The high cytotoxicity of YM155 was
18 completely abolished by knockout of *SLC35F2* (Fig. 2), and the induction of YM155
19 resistance by knockout of *SLC35F2* was useful for enriched selection of genome-
20 edited clones (Figs. 4 and 5). Live monitoring revealed that un-targeted cells
21 underwent cell death in response to YM155 (Fig. S2F, Movies S2 and S3).
22 Considering the undesirable biases that could result from permanent knockout of
23 *SLC35F2* (Fig. 5), we then attempted to temporarily induce YM155 resistance by
24 transient knockdown of *SLC35F2*, and we found that this was sufficient to achieve
25 enriched selection of targeted clones (Fig. 6). The function of *SLC35F2* in the edited

1 clones was fully recovered (Fig. 6). Following this scarless YES-approach, CRISPR-
2 targeted clones were achieved with up to 72.2% efficiency, avoiding the need for
3 laborious clonal selection within 3 weeks.

4 It is important to note that YM155 sensitivity varies depending on cell type
5 due to differences in the expression level of *SLC35F2* (Fig. S2A) as well as the
6 sensitivity of the DNA damage response. The high expression level of *SLC35F2* in
7 most hPSCs (Kolle et al., 2009) (Fig. 1C) may render the YES-approach uniquely
8 suitable for application in these cells. In addition, the strong mitochondria-dependent
9 apoptotic cell death response of hPSCs by genotoxic stresses (Liu et al., 2013), which
10 depends on mitochondrial translocation of p53 (Lee et al., 2013b), may contribute to
11 the high selectivity of YM155: as previously reported (Winter et al., 2014),
12 intracellular YM155 taken up through SLC35F2 induced a clear DNA damage
13 response (Fig. 1G). Unlike other chemical-based selection approaches, induced
14 YM155 resistance was achieved by abrogating a drug-specific membrane importer,
15 thereby blocking cellular uptake of YM155. Thus, due to their failure to take up
16 YM155, clones that survived enriched selection would be free from undesirable
17 effects of YM155.

18 However, the scarless YES-approach requires colony selection to avoid false-
19 positive clones (Fig. 5A) that could survive YM155 treatment. Notwithstanding,
20 development of a one-vector system for expression of siRNA and Cas9 would
21 minimize the incidence of false-positive or -negative clones. Taken together, our
22 findings indicate that the scarless YES-approach could be further developed as a
23 useful tool for advancing the application of genome engineering in hPSCs. In
24 particular, this strategy would enable the use of sgRNA libraries to establish whole
25 panels of hPSCs that mimic genetic diseases.

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4

5 **Author Contributions**

6 HJ.C conceived the overall study design and led the experiments. KT.K and JC.P
7 mainly conducted the experiments, data analysis, and critical discussion of the results.
8 H.L and W.K conducted the informatics analysis. HK.J and H.K and SS.B contributed
9 to sgRNA design and NGS analysis. Y.J and J.L measured the intracellular level of
10 YM155. All authors contributed to manuscript writing and revising, and endorsed the
11 final manuscript.

12

13 **Declaration of Interests**

14 The authors declare no competing interests.

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35

1 **Figure legends**

2 **Figure 1. High expression of *SLC35F2* mediates intracellular uptake of YM155**

3 **in hPSCs** (A) A graphical schema of correlating cell line hPSC scores with cell line
4 drug responses (AUC) against each of 543 compounds available in CTRP (B) Left,
5 the correlation strength (z-scored Pearson correlation coefficient) of 543 compounds
6 from comparing AUCs of each compound to hPSC scores, Cells with high hPSC
7 scores (hPSC-like cells) are selectively sensitive to YM155. Right, the correlation
8 strength of 18,858 genes from comparing the expression values of each gene with
9 AUCs of YM155. *ATP1B1* and *SLC35F2* are highly expressed in YM155 resistant
10 and sensitive cells, respectively. (C) Expression of *SLC35F2* in several hESC cell
11 lines from NextBio portal, Differentiated cells (blue dots) were analyzed as controls.
12 (P.C. indicates PC-3 prostate cancer cell). (D) mRNA expression of *SLC35F2* and
13 *POU5F1* of human dermal fibroblasts (hDF), hESC-derived MSCs and hESCs (E)
14 mRNA expression levels of *SLC35F2* and *POU5F1* of hESC-MSCs, hCHA3, H9 and
15 SES8 iPSCs. (F) Fluorescent images of H9 stained with γ H2AX after YM155
16 treatment, DAPI for nuclear counterstaining (scale bars = 10 μ m) (G) Immunoblotting
17 assay between hESC-MSCs and hESCs were performed to detect pH2AX (Ser 139)
18 and cleaved Caspase 3 (c-Casp 3) level after YM155 treatment. α -tubulin was used
19 for internal control. (H) LC-MS/MS analysis was performed to quantify the
20 intracellular YM155 amount between hDF and hESCs. 1 μ M of YM155 was treated
21 for 2 hours.

22

23 **Figure 2. *SLC35F2* enables YM155-induced selective cell death in hPSCs** (A)

24 Scheme for *SLC35F2* knockout targeting Exon 7 (B) Experimental schema of
25 enrichment of *SLC35F2* KO hESCs (top panel), Flow cytometry of Annexin V and

1 microscopic images of hESCs after 50nM of YM155 treatment (Red arrow indicates
2 YM155 resistant clone: YM155R, scale bars = 500 μ m) (C) T7E1 assay with sgRNA
3 of SLC35F2 and Cas9 transfected hESCs after treatment of indicative dose of YM155,
4 Indel percentage shown as below (D) Percentage of mutation quantified from NGS
5 analysis was graphically presented. (E) Sequence information from a YM155R clone
6 (KO #1), sgRNA target sequences and PAM sequence were shown in Red and Orange
7 respectively. (F) Flow cytometry of Annexin-V apoptosis assay in WT (NC) and
8 SLC35F2 KO#1 hESCs after indicative dose of YM155 treatment (G) Intracellular
9 level of YM155 in control (NC) and KO hESCs (KO#1) determined by LC-MS/MS
10 (H) Fluorescent microscopic images of hESCs stained with γ H2AX in control (NC)
11 and SLC35F2 KO (KO#1) hESCs with DMSO as a vehicle or 20 nM of YM155
12 treatment, DAPI for nuclear counterstaining (scale bars = 10 μ m)

13

14 **Figure 3. *SLC35F2* KO hPSCs maintain pluripotency and proliferation intact** (A)
15 Relative mRNA expression of indicative pluripotent markers in human dermal
16 fibroblast, and control (NC) and SLC35F2 KO (KO#1) hESCs (B) Immunoblotting
17 analysis for indicative proteins (SOX2 and OCT4 in control (NC) and SLC35F2 KO
18 (KO#1) hESCs, β -actin was used as a loading control. (C) Alkaline phosphatase
19 activity assay of control (NC) and SLC35F2 KO (KO#1) hESCs, Insets are enlarged
20 images of representative colonies, respectively. (D) Cell proliferation rate of control
21 (NC) and SLC35F2 KO (KO#1) hESCs was graphically presented. (E) Flow
22 cytometry of green fluorescence of EGFP expressing control and SLC35F2 KO
23 (KO#1) hESCs determined at indicative days (F) Relative levels of mRNA expression
24 of typical lineage specific genes (endoderm: *SOX17*, *GATA6*, mesoderm: *MSX1* and
25 ectoderm: *NESTIN*) of control (NC) and SLC35F2 KO (KO#1) hESCs at indicative

1 days after *in vitro* differentiation (G) Images of typical three germ layers from the
 2 teratoma stained with H&E staining, Masson's trichrome, and Alcian Blue. The scale
 3 bar represents 50 mm. (H) Scatter plot of whole genes from control (NC) and
 4 SLC35F2 KO (KO#1) hESCs, Typical pluripotent marker genes (*POU5F1*, *SOX2*,
 5 *NANOG*, *Lin28A*) were marked as red circles.

6

7 **Figure 4. YM155 mediated enriched selection** (A) Graphical schema for YM155
 8 mediated enriched selection approach in HEK293T cells (GOI: Gene-of-interest) (B)
 9 T7E1 assay of control (NC) and SLC35F2 KO HEK293T cells, The asterisk indicates
 10 a cleaved band after T7E1 enzyme treatment. (C) NGS data of control (NC) and
 11 SLC35F2 KO (KO#1) HEK293T cells, Indel variety of control (NC) and SLC35F2
 12 KO (KO) HEK293T cells was graphically presented. (D) A graphical summary of
 13 surrogate vector system for CCR5 target (E) Fluorescence microscopic images of
 14 HEK293T cells expressing surrogate reporter after sgRNA transfection of *CCR5* only
 15 (*CCR5* only) or *CCR5/SLC35F2* (*CCR5*+*SLC35F2*) with or without YM155
 16 treatment, Ratio of GFP over mRFP positive population was shown in below.
 17 Average of ratio of GFP over mRFP positive population at indicative conditions (TS:
 18 Surrogate reporter only, C: sgRNA for *CCR5*, S: sgRNA for *SLC35F2*,
 19 *CCR5*+*SLC35F2*: sgRNA for both *CCR5* and *SLC35F2*) was graphically presented.
 20 (G) T7E1 assay for *CCR5* and *SLC35F2* at indicative conditions (TS: Surrogate
 21 reporter only, C: sgRNA for *CCR5*, S: sgRNA for *SLC35F2*, *CCR5*+*SLC35F2*:
 22 sgRNA for both *CCR5* and *SLC35F2*), The asterisk indicates a cleaved band after
 23 T7E1 enzyme treatment. Relative band densities were shown in below.

24

Figure 5. YM155 based enriched selection of genome-edited hESCs (A) T7E1 assay for CCR5 and SLC35F2 in each clone of hESCs (Seven clones for C:S=1:1, and four clones for C:S=2:1) at indicative conditions {different dose ratio of sgRNA of CCR5(C) vs SLC35F2(S)} (left panel), Summary table of number of KO clones of CCR5 and SLC35F2 (right panel) (B) NGS result from #1 clone (from C:S=1:1) compared to control (WT control), sgRNA target and PAM sequence were shown in Green and Red letters respectively. (C) Clustering of RNA-seq samples using t-distributed stochastic neighbor embedding (t-SNE) based on the expression of whole genes, hPSC signature genes, and cellular transition metal ion homeostasis (GO:0046916) genes.

Figure 6. Scarless YES-approach for establishment of CCR5 targeted hESCs (A) Graphical schema of scarless YES-approach (B) Relative levels of mRNA expression of *SLC35F2* at indicative days after siRNA transfection in hESCs (C) Flow cytometry of Annexin V and 7AAD staining at 2 or 5 days after transfection of control siRNA (siNC) and siRNA for SLC35F2 (siSLC35F2) with indicative dose of YM155 treatment (D) Average of ratio of indel in CCR5 after YES-approach at indicative dose of YM155 treatment (E) Deep sequencing analysis of CCR5 KO clones achieved by YES-approach at indicative dose of YM155 treatment (F) NGS data for CCR5 target sequence, sgRNA target and PAM sequence were shown in Green and Red respectively. (G) Flow cytometry of Annexin-V/7-AAD staining at indicative dose of YM155 in WT or CCR5 KO hESCs

1 **STAR Methods**

2 **Pharmacogenomics data sets**

3 Basal-level cell line mRNA expression data was obtained from the Cancer
4 Cell Line Encyclopedia (CCLE) data portal
5 (<https://portals.broadinstitute.org/ccle/data>) (Barretina et al., 2012). Raw Affymetrix
6 CEL files were converted to normalize real expression value for each gene probe set
7 using Robust Multi-array Average (RMA) method. When collapsing probes to genes,
8 the maximum of expression values of multiple probes per gene was taken as a
9 representative expression value for the gene. Among the total 886 cell lines, we
10 excluded hematologic lineages, non-human, and ambiguous tissue types, and used
11 666 cancer cell lines with drug sensitivity data in this analysis. Cell line drug response
12 data was downloaded from Cancer Therapeutics Response Portal
13 (<https://ocg.cancer.gov/programs/ctd2/data-portal>) (Seashore-Ludlow et al., 2015).
14 Cell viability values were adjusted to a range of 0-100 % and fitted by 4-parameter
15 logistic regression analysis. We selected a unique range of concentrations that tested
16 on the most cells for each compound to calculate AUC (area under the fitted curve).
17 AUC was normalized to a range of 0-1 by the maximum AUC assumed to be 0 %
18 growth inhibition in a given concentration range.

19

20 **Cell line and culture**

21 Human embryonic stem cells (WA09, WiCell Research Institute) were
22 cultured on Matrigel (BD Biosciences) coated dishes fed with mTeSRTM-E8TM
23 media (STEMCELL technologies) and StemMACSTM media (Miltenyi-Biotec)
24 added with 50 µg/ml Gentamicin (Life Technologies). Cells were passaged every 5-6
25 days and media was changed every day. hESC were rinsed with DPBS and exposed to

1 Dispace (Gibco) for detachment. Detached cells were washed with DMEM/F-12
2 (Gibco) media and plated on Matrigel-coated plate. 10 μ M of Y27632 (Gibco) was
3 added for cell attachment when needed. HEK293T cells were cultured on a culture
4 dish (Falcon) fed with DMEM (Gibco) added with 10% FBS (Gibco) and 50 μ g/ml
5 Gentamicin. Upon transfer, HEK293T cells were rinsed with DPBS and detached
6 enzymatically with 0.25% Trypsin. Trypsin was inactivated by adding DMEM
7 containing 10% FBS and appropriate cells were seeded on the dishes.

8

9 **Teratoma formation assay**

10 After anesthesia, direct testicular injection of WT, *SLC35F2* KO hESCs was
11 performed to examine in vivo pluripotency of each cell. 5x10⁶ cells of each WT (n=3)
12 and *SLC35F2* KO (n=3) hESC were inoculated subcutaneously and intracutaneously
13 to mice with 4-weeks-old. Mice were euthanized 2 months after inoculation of each
14 WT and *SLC35F2* KO hESC. These animal experiments were conducted under the
15 permission of Seoul National University Institutional Animal Care and Use
16 Committee (permission number: SNU-180810-1).

17

18 **RNA-sequencing analysis**

19 Total RNA was extracted from cells using Easy-BLUETM RNA isolation kit
20 (iNtRON Biotechnology) and quality control was performed. The qualified samples
21 proceed to library construction. The sequencing library is prepared by random
22 fragmentation of the DNA or cDNA sample, followed by 5' and 3' adapter ligation.
23 The read quality of raw RNA-seq files (FASTQ) was checked using FastQC (v0.11.7)
24 and the adapter sequences in reads were eliminated using cutadapt (v1.8.1). Trimmed
25 FASTQ files were aligned to GRCh38 genome using STAR aligner (v2.6.0a), then the

1 read counts for genes were acquired using HTseq (v0.11.0) with Gencode v22
2 annotation GTF (https://www.gencodegenes.org/human/release_22.html).

3

4 **LC-MS/MS analysis**

5 HEK293T and H9 cells were exposed to YM155 for 1 hour, cells were 1x10⁶
6 cells were counted. Harvested cells were lysed with 80% methanol and incubated in
7 ice for an additional 1 hour. After spin down with 13,000 rpm for 20 minutes,
8 supernatants were collected and evaporated with N₂ gas until no more solvents
9 remained. Sample residues were re-suspended with 100 µL of 50% methanol followed
10 by 10 seconds sonication, 5 seconds vortex, spin-down and filtration with 0.2 µm
11 membrane filter. Total 5 µL of solvents were injected to UHPLC/Q-TOF MS (Waters,
12 Milford, MA, USA) using ACQUITY UPLC BEH C18 column (50 x 2.1 mm, 1.7
13 mm, 30 °C). Samples were read with 0.2 mL/ min flow rate and the estimated peak
14 for YM155 was 4.32 ~ 4.9 min.

15

16 **T7E1 assay**

17 PCR is performed using SolgTM Taq DNA Polymerase (STD16-R500,
18 SolGent), following the supplier's instruction with 10 µl of total volume per sample.
19 1st PCR is performed with primer F1 and primer R for each gene. The 1st PCR
20 product is diluted in 190 µl of DW. 2nd PCR is performed with 1 µl of diluted 1st
21 PCR product, using primer F2 and primer R for each gene. The 2nd PCR product is
22 mixed with the same volume of 2X NEBuffer2 (B7002S, New England BioLabs) and
23 hybridized. 3 units of T7E1 endonuclease (M0302S, New England BioLabs) were
24 treated to 10 µl of hybridized 2nd PCR product. The enzyme reaction is conducted for
25 40 minutes at 37 °C water bath.

1

2 **Flow cytometry**

3 Cells were detached with Accutase™ (561527, BD Biosciences) and washed
4 with DPBS for three times and then stained with FITC Annexin-V (556419, BD
5 Biosciences) and 7-AAD (559925, BD Biosciences) for cell death detection. Diluted
6 1X Annexin V binding buffer (556454, BD Biosciences) is used as the solvent for
7 staining. FACScalibur from BD Biosciences and CellQuest software was used for
8 FACS analysis.

9

10 **Statistical analysis**

11 The quantitative data are expressed as the mean values $\pm$ standard error of the
12 mean (SEM). Student's paired t-tests or one-way ANOVAs were performed to
13 analyze the statistical significance of each response variable. Pre-specified
14 comparisons between groups were conducted (when appropriate) by Tukey's post hoc
15 test using the SPSS program (Statistical Package for the Social Sciences, version 17).
16 p values less than 0.05 were considered statistically significant.

17

18

19

20

Figure. 1

Figure. 2

Figure. 3

Figure. 4

Figure. 5

Figure. 6