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Neurod1 is essential for the survival and maturation of adult-born neurons

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Abstract

The transcriptional program that controls adult neurogenesis is unknown. We generated mice with an inducible stem cell–specific deletion of *Neurod1*, resulting in substantially fewer newborn neurons in the hippocampus and olfactory bulb. Thus, *Neurod1* is cell-intrinsically required for the survival and maturation of adult-born neurons.

The ability to generate new neurons provides the brain with an important level of plasticity for maintaining cellular homeostasis and potentially underlies a response to injury. Studies of adult neurogenesis have revealed extrinsic signals controlling the fate of resident neural stem cells[1]. However, the cell-intrinsic transcription factors required to generate and promote the survival of newborn neurons have not been fully elucidated. During development, the proneural basic helix-loop-helix (bHLH) transcription factors are essential for embryonic neurogenesis[2]. To identify genes required for adult neurogenesis, we focused on the bHLH transcription factor *Neurod1* (also known as *NeuroD* or *Beta2*)[3,4]. *Neurod1* is predominantly expressed in the nervous system late in development and is therefore more likely to be involved in terminal differentiation, neuronal maturation and survival[3,5,6]. Because *Neurod1*-null mice die perinatally from neonatal diabetes as a result of the loss of *Ins* gene expression[7], we used an inducible gene ablation approach and found that *Neurod1* is necessary for newborn granule cells to survive and fully mature in the adult mammalian brain.

To define the cells in the hippocampus subgranular zone (SGZ) that express *Neurod1*, we stained for NeuroD1 and a variety of cell type–specific markers in *nestin* promoter–driven GFP transgenic reporter mice[8] (Fig. 1 and Supplementary Fig. 1). NeuroD1 was not expressed in the type 1 (nestin-GFP and GFAP double positive) radial stem-like cell (Fig.

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AUTHOR CONTRIBUTIONS

Z.G., K.U. and J.H. conducted the experiments, contributed to the data analyses and designed the study. J.L.A. contributed to the Neurod1 expression analysis, K.-A.N. and S.G. provided the *Neurod1*$^{loxP/loxP}$ mice, and A.J.E. and D.C.L. provided *Nes-CreER*^{T2} mice and contributed conceptually to the project. The manuscript was written by J.H. and commented on by all authors.

1a) and was first detected in ~20% of type 2a (nestin-GFP positive, GFAP negative and DCX negative) progenitors (Fig. 1a). NeuroD1 colocalized with Ki67, a marker of dividing cells, in a small subset of progenitors (Fig. 1a) and did not colocalize with the majority of Sox2-positive cells, although we detected rare cells expressing both NeuroD1 and Sox2 (Supplementary Fig. 2). NeuroD1 expression peaked in 490% of type 2b (nestin-GFP and DCX double positive) progenitors and in ~100% of type 3 (DCX positive only) neuroblasts (Fig. 1a). NeuroD1 also overlapped with the homeobox transcription factor Prox-1, which labels immature and mature granule neurons, and was downregulated in mature NeuN-expressing granule neurons (Fig. 1a). These data suggest that NeuroD1 has a transient and dynamic expression profile that is restricted to progenitors/neuroblasts (type 2b and 3 cells) as they transition to immature granule neurons.

To study the role of *Neurod1* in adult neurogenesis, we ablated *Neurod1* in the neurogenic compartments of the adult brain: the subventricular zone (SVZ) and the SGZ. We crossed mice harboring a tamoxifen (TAM)-inducible form of Cre recombinase ($CreER^{T2}$) under nestin transcriptional control[9] with $Neurod1^{loxP/loxP}$ mice[10], combined with the *Rosa26* (*R26R*)-*yfp* reporter line, to achieve deletion of *Neurod1* in all nestin-expressing stem cells and their progeny (NeuroD1 cKO; Supplementary Fig. 3 and Supplementary Methods).

To determine the dynamics of *Neurod1* expression in recombined YFP-positive cells, $Nes\text{-}creER^{T2}$; *R26R-yfp* mice were killed at different time points after TAM treatment (Fig. 1c). About 10% of the YFP-positive cells were positive for Neurod1 by 1 d after TAM treatment, which increased to ~40% at 8 d post TAM treatment, mostly in type 2 and 3 cells (Fig. 1c). The percentage of YFP and Neurod1 double-positive cells reached a plateau after 8 d, consistent with an earlier study[9].

In light of the *Neurod1* expression dynamics, we chose two time points, 6 and 40 d post TAM treatment, for further analysis. Wild-type littermates (controls) and NeuroD1 cKO mice were killed at the indicated time points post TAM treatment. At both time points, we found successful Cre-mediated recombination and deletion of *Neurod1* in NeuroD1 cKO mice (wild type, 3,216 ± 1,551; cKO, 510 ± 218 YFP and Neurod1 double-positive cells; Fig. 1b, Supplementary Fig. 2 and data not shown). We quantified the number of YFP-positive cells in the SGZ and found no difference between NeuroD1 cKO and wild-type mice at 6 d post TAM treatment (Fig. 1b,d), suggesting that *Neurod1* is not required in early stem/progenitor cells. In contrast, there was a substantial decrease in the number of YFP-positive cells between wild-type and NeuroD1 cKO mice at 40 d post TAM treatment (Fig. 1b,d). Morphological assessment of these YFP-positive cells showed selective loss of newborn granule neurons, without changing the stem/progenitor cell pool (Fig. 1e and Supplementary Fig. 4), suggesting that *Neurod1* is required in late-stage progenitors as they differentiate into hippocampal granule neurons but is dispensable in early stem/progenitor cells.

Consistent with our morphological analyses, the number of Sox2, GFAP and YFP triple-positive (Type 1), Sox2 and YFP double-positive, or Ascl1-positive (a proneural bHLH factor upstream of *Neurod1*) cells did not change between wild-type and NeuroD1 cKO mice at 40 d post TAM treatment (Fig. 2 and Supplementary Fig. 5). In addition, proliferation of YFP-positive cells (Ki67- and BrdU-positive cells) (data not shown) remained unchanged between the two groups at 6 d post TAM treatment (Fig. 2b,d). In contrast, there was a marked decrease in the number of YFP and Prox-1 double-positive (Fig. 2c,d) and YFP and DCX double-positive cells (Supplementary Fig. 6) at 40 d post TAM treatment. The deletion of *Neurod1* could decrease the number of YFP-positive cells by halting the progression of progenitors/immature neurons to mature granule cells. Indeed, wild-type mice had mature YFP, Prox-1 and NeuN triple-positive granule neurons at 40 d

post TAM treatment, which showed a decreasing trend in NeuroD1 cKO mice (Fig. 2c,d). Consistent with this, we observed a reduction, albeit to a lesser extent compared with what we observed at 40 d, of YFP-positive cells in NeuroD1 cKO mice compared with wild-type mice at an intermediate time point of 18 d post TAM treatment (Supplementary Fig. 7). These results suggest that *Neurod1* is important in the differentiation of progenitors to immature and mature neurons, similar to a recent report[11].

The decrease in YFP-positive granule neurons in NeuroD1 cKO mice could also be a result of defects in the survival and/or the maturation of newborn granule cells. Notably, we found more active caspase 3 (AC3) positive cells at 40 d, but not at 6 or 18 d, post TAM treatment in NeuroD1 cKO mice (227 ± 85 cells) compared with wild types (77 ± 24 cells) (Fig. 2e and data not shown). Closer examination revealed that AC3-positive cells in the dentate gyrus of NeuroD1 cKO and wild-type mice did not colocalize with DCX at 40 d post TAM treatment, although AC3 and DCX double-positive cells were readily observed at 18 d post TAM treatment (Supplementary Fig. 8), suggesting that the death of newborn neurons is a transient event and/or the increase of dead/dying cells occurs after the DCX-positive immature neuron stage. Furthermore, we observed a reduction in dendritic lengths of YFP-positive granule neurons in NeuroD1 cKO mice (363 ± 72 μm) compared with wild types (598 ± 116 μm), which is consistent with early studies describing dendritic defects in the absence of *Neurod1* (Fig. 2f)[6,12]. Together, these data suggest that *Neurod1* functions cell intrinsically in progenitors/immature neurons to promote their survival and maturation into mature granule neurons.

To examine whether *Neurod1* is involved in olfactory bulb neurogenesis, we looked for YFP and Neurod1 double-positive cells in the SVZ, rostral migratory stream (RMS) and olfactory bulb in wild-type mice (Supplementary Fig. 9). Notably, YFP and Neurod1 double-positive cells were only detected in the caudal RMS and were rarely seen in the SVZ and olfactory bulb, suggesting that *Neurod1* is involved in the migration of progenitors as they differentiate into olfactory bulb neurons. Because of the density of cells in the RMS, we quantified YFP-positive cells in the olfactory bulb glomerular cell and granule cell layers. The number of YFP-positive cells decreased in the glomerular cell and granule cell layers of NeuroD1 cKO mice at 40 d post TAM treatment compared with wild types (Supplementary Fig. 10). These data suggest that *Neurod1*'s function is also important for promoting the survival and differentiation of adult-born olfactory bulb neurons.

To further delineate the role of *Neurod1* in adult neurogenesis, we prepared hippocampus and SVZ neurospheres from wild-type and NeuroD1 cKO mice and infected cells with adenoviruses expressing GFP (control) or Cre-GFP. We observed a decrease in Tuj1-positive neurons in Cre-GFP–infected NeuroD1 cKO cells (28 ± 1.2%) after neuronal differentiation compared with GFP-infected cells (41 ± 7%) (Fig. 3a). In contrast, there was no change in GFAP-positive astrocytes between Cre-GFP–infected NeuroD1 cKO cells (62 ± 12%) and wild-type cells (65 ± 6%) (Fig. 3b). Moreover, wild-type cells over-expressing a full-length *Neurod1* cDNA showed an increase in Tuj1-positive neurons (from 0 to 70 ± 12%) under proliferating conditions (Fig. 3c). *Neurod1* and *Tuj1* mRNA levels decreased in Cre-GFP–infected NeuroD1 cKO cells (Fig. 3d). In the absence of *Neurod1*, there was a decrease in the expression of neuronal/pro-survival genes, whereas the expression of *Gfap* was not dramatically altered (Fig. 3d). Thus, *Neurod1* is both necessary and sufficient to promote neuronal differentiation and maturation *in vitro*, consistent with its essential role *in vivo*.

Using a genetic approach in mice, we found that *Neurod1* is a cell-intrinsic factor required for the survival and differentiation of newborn neurons in the adult SGZ and SVZ. Recent studies have shown that a wide variety of stimuli, including seizures, running and enriched

environment, can profoundly induce adult neurogenesis[1]. An exciting possibility is that these stimuli may act, in part, via *Neurod1* target genes to control the survival and maturation of newborn neurons[13]. Although the cause-and-effect relationship between cell death and failure to mature has not been fully established, our results suggest that *Neurod1* is not only critical for the survival and differentiation of adult-born granule neurons but also for their maturation and integration into the neuronal circuitry. These studies suggest that *Neurod1* is a critical regulator of adult neurogenesis and may serve as a potential therapeutic target in regenerative medicine and in the prevention of age-related cognitive decline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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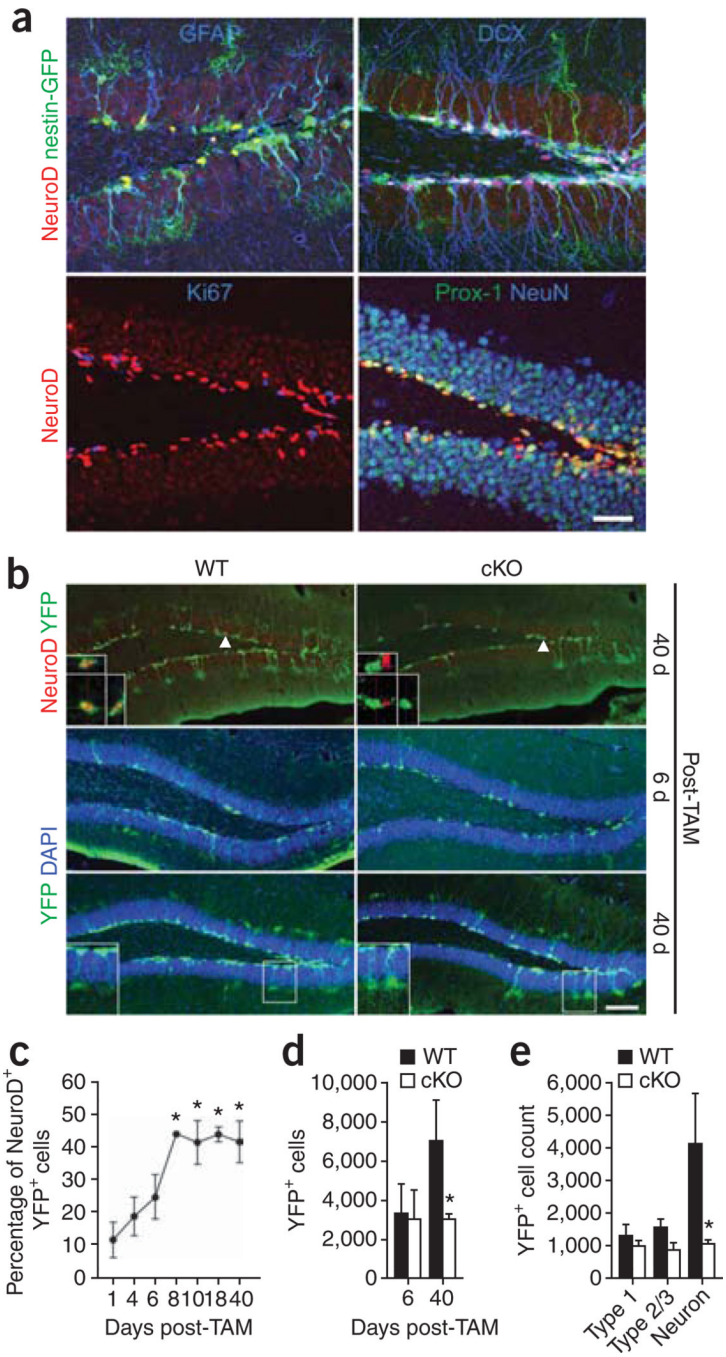

Figure 1. Critical role of *Neurod1* in adult hippocampal neurogenesis

(**a**) Immunostaining of dentate gyrus of nestin-GFP mice. Scale bar represents 50 μm. (**b**) Immunostaining of YFP-positive cells (green) and Neurod1 (red) in dentate gyrus of wild-type (WT) and NeuroD1 cKO mice at 40 d post TAM treatment (top). Arrowheads correspond to insets of enlarged orthogonal images of YFP and NeuroD1 double-positive cells in wild-type mice and YFP-positive and NeuroD1-negative cells in NeuroD1 cKO mice, respectively. We immunostained the dentate gyrus of wild-type and NeuroD1 cKO mice for YFP (green) and DAPI (nuclear counterstain, blue) at 6 and 40 d post TAM treatment (bottom). Bottom insets show enlarged images of YFP-positive cells that are type

1 with radial stem-like morphology. Scale bar represents 100 μm. (**c**) Time course analysis of the percentage of YFP and Neurod1 double-positive cells in wild-type mice (* $P < 0.01$ versus 1 d, ANOVA with Bonferroni post hoc, data represent ± s.d. from $n = 3$ mice). (**d**) Quantification of YFP-positive cells in the dentate gyrus of wild-type and NeuroD1 cKO at 6 and 40 d post TAM treatment (* $P < 0.001$, *t* test, data represent ± s.d. from $n = 8–10$ per group). (**e**) Number of YFP-positive cells in dentate gyrus that are type 1 and 2/3 cells or granule neurons 40 d post TAM treatment of wild-type and NeuroD1 cKO mice (* $P < 0.001$, *t* test, data represent ± s.d., $n = 6–8$ per group).

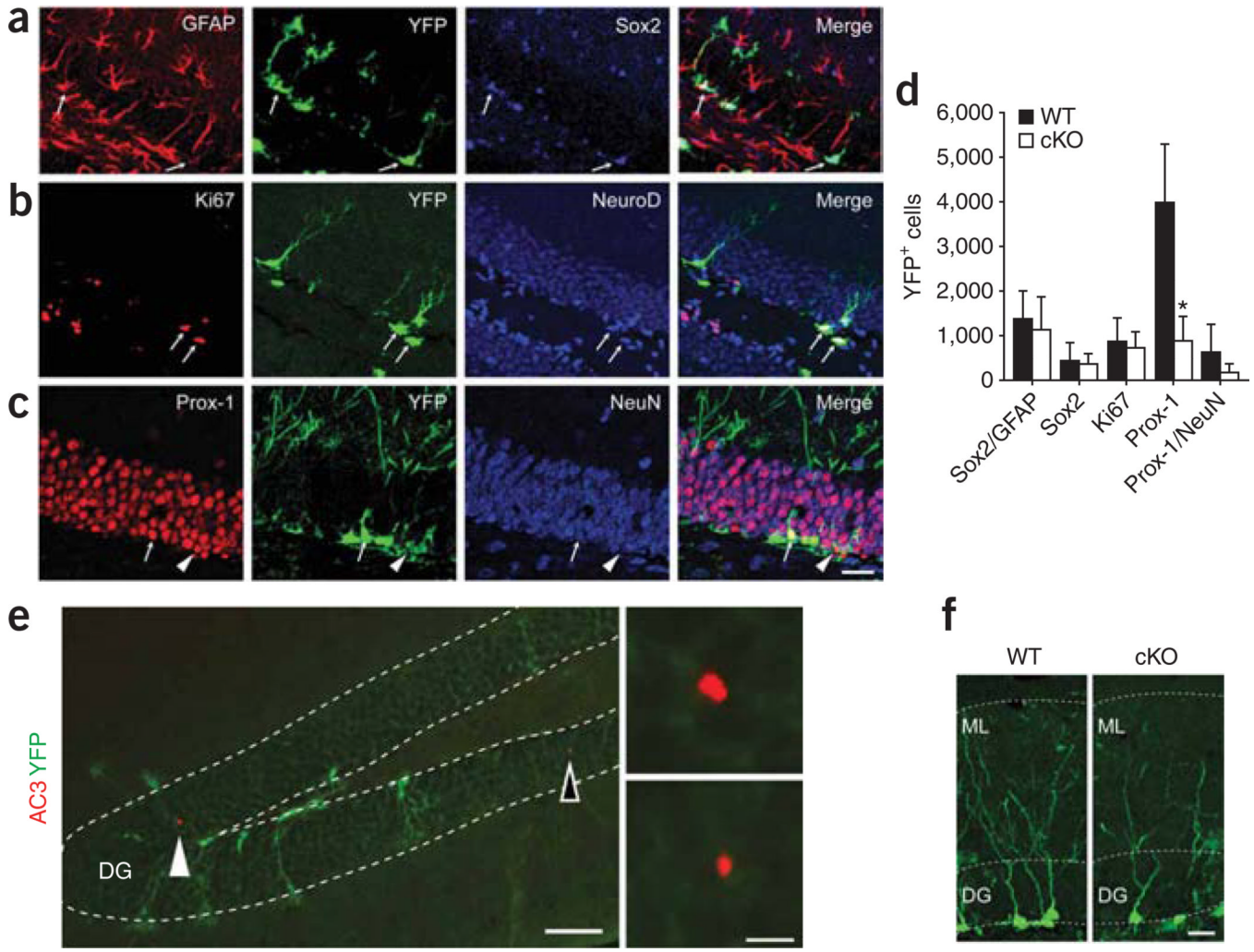

Figure 2. ***Neurod1*** **is required for the survival and maturation of adult-born granule cell neurons**

(**a**) Type 1 radial stem-like cells expressed GFAP, YFP and Sox2. Arrows indicate triple-labeled cells in NeuroD1 cKO mice. (**b**) Representative image of Ki67, YFP and Neurod1 triple-positive cells (arrows) in the dentate gyrus of NeuroD1 cKO mice. (**c**) An example of a YFP and Prox-1 double-positive immature granule neuron (arrowhead) and a YFP, Prox-1 and NeuN triple-positive mature granule neuron (arrow) in NeuroD1 cKO mice. Scale bar represents 25 μm. (**d**) Quantification of YFP-positive cells expressing various markers in the dentate gyrus of wild-type and NeuroD1 cKO mice at 40 d post TAM treatment (* $P <$ 0.001, *t* test, data represent ± s.d., $n = 4–6$ per group). (**e**) A representative image of two AC3-positive cells. Arrowheads represent enlarged insets of dentate gyrus of NeuroD1 cKO mice (white, top panel; black, bottom panel). Scale bars represent 100 μm and 15 μm (insets). (**f**) Dendrites of YFP-positive adult-born neurons. Shown are sample projections of *Z* series confocal images of YFP-positive neurons. Scale bar represents 20 μm. DG, dentate gyrus; ML, molecular layer.

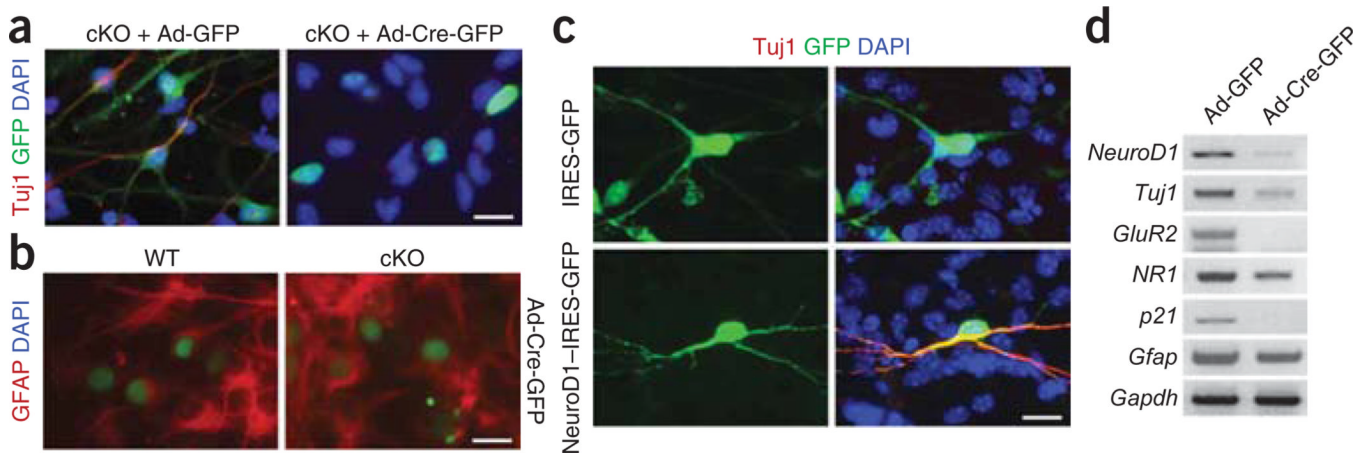

Figure 3. ***Neurod1*** **is required for neuronal, but not astrocyte, differentiation in neurosphere cultures**

(**a**) Immunostaining for the neuronal marker Tuj1 (red) and adenovirus expressing control GFP or Cre-GFP (green) in SGZ and SVZ neurospheres from NeuroD1 cKO mice. (**b**) Immunostaining for GFAP (red) and adenovirus expressing Cre-GFP (green) in SGZ and SVZ neurospheres from wild-type and NeuroD1 cKO mice. (**c**) Immunostaining for Tuj1 (red) and IRES-GFP or *Neurod1*–IRES-GFP (green) in SGZ and SVZ neurospheres from wild-type mice. DAPI is stained in blue. Scale bars represent 10 μm. (**d**) RT-PCR analysis of NeuroD1 cKO neurospheres under neuronal differentiation conditions after infection with adenovirus expressing GFP or Cre-GFP. *Gapdh* is used as a normalization control.