A regulatory network involving Foxn4, Mash-1 and Delta-like 4/Notch-1 generates V2a and V2b spinal interneurons from a common progenitor pool

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Running title: Foxn4, Dll4/Notch-1 and Mash-1 specify V2a/V2b spinal interneurons

SUMMARY (188 words)

In the developing central nervous system, cellular diversity depends in part on organizing signals that establish regionally-restricted progenitor domains, each of which produces distinct types of differentiated neurons. However, the mechanisms of neuronal sub-type specification within each progenitor domain remain poorly understood. The p2 progenitor domain in the ventral spinal cord gives rise to two interneuron subtypes called V2a and V2b, which integrate into local neuronal networks that control motor activity and locomotion. Foxn4, a forkhead transcription factor, is expressed in the common progenitors of V2a and V2b interneurons and is required directly for V2b but not for V2a development. We show here that Foxn4 induces expression of Delta-like 4 (Dll4) and Mash-1/Ascl1. Dll4 then signals through Notch-1 to subdivide the p2 progenitor pool. Foxn4, Mash-1/Ascl1 and activated Notch-1 trigger the genetic cascade leading to V2b interneurons while the complementary set of progenitors, without active Notch-1, generates V2a interneurons. Thus Foxn4 plays a dual role in V2 IN development: 1) by initiating Delta-Notch signalling, it introduces the asymmetry required for development of V2a and V2b INs from their common progenitors; 2) it simultaneously activates the V2b genetic programme.

INTRODUCTION

The neurons and glial cells of the mature central nervous system (CNS) develop from the neuroepithelial cells that surround the lumen of the embryonic spinal cord and the ventricles of the

Chx10 (Ericson et al., 1997), whereas V2b INs express transcription factors Gata2, Gata3 and Scl (Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002). How V2 INs incorporate into the local spinal circuitry is not established, although V2a INs are known to be excitatory (glutamatergic) and to project ipsilaterally (Kiehn, 2004; Kimura et al., 2006). The neurotransmitter phenotype of V2b INs is not known. V2a and V2b INs are derived from common progenitors that initially express the forkhead/ winged helix transcription factor *Foxn4* (Li et al., 2005 and this paper). How does this homogeneous progenitor pool generate two distinct neuronal sub-types?

The Notch-Delta signalling pathway is often used to establish or to maintain differences between lineally related cells (Artavanis-Tsakonas et al., 1999; Louvi et al., 2006). For example, signalling between Notch1 and its ligand Delta-like 4 (Dll4) in endothelial cells is necessary for artery-vein discrimination and also for sprouting of lymphatic vessels from veins (Duarte et al., 2004; Seo et al., 2006). We thought it possible that the distinction between V2a and V2b INs might also be established through Notch-Delta signalling. Notch1-3 are all expressed in the ventral VZ of the embryonic spinal cord (Lindsell et al., 1996), as are their ligands Dll1, Dll3, Dll4 and Jagged (Benedito and Duarte, 2005; Dunwoodie et al., 1997; Lindsell et al., 1996; Mailhos et al., 2001). Unlike Dll1 and Dll3, which are expressed widely throughout the VZ and/or in postmitotic neurons, Dll4 appears to be restricted to the p2 domain of the VZ, suggesting a specific role in V2 interneuron development (Benedito and Duarte, 2005).

We have examined the relationship between Foxn4 and Notch-Delta signalling during development of V2a and V2b sub-lineages. We demonstrated that Foxn4 is a master regulator of the V2b sub-lineage, being necessary and sufficient to induce the V2b determinants Gata2, Gata3 and Scl. We also found that Foxn4 controls *Dll4* and *Mash-1/Ascl-1* expression in p2. In gain of function assays, Dll4 inhibited the development of V2a INs and conversely, when Notch-1 was conditionally inactivated in Nestin-expressing progenitor cells and their derivatives, V2a INs were over-produced at the expense of V2b INs. Taken together, our data suggest the following model: 1) Foxn4 activates *Dll4* and *Mash-1/Ascl1* in common V2a/V2b progenitors; 2) subsequent neighbour-to-neighbour signalling via Dll4 activates Notch-1 in a subset of p2 progenitors, which then generate V2b INs under the combined action of Notch-1, Foxn4 and Mash-1; 3) the complementary set of progenitors fails to activate Notch-1 and consequently generates V2a INs.

RESULTS

Foxn4 is necessary and sufficient for V2b lineage specification, and suppresses V2a INs

Foxn4 expression has been described in the developing mouse retina and neural tube (Gouge et al., 2001; Li et al., 2004; Li et al., 2005). In the ventral neural tube it is expressed specifically in the p2 progenitor domain (Li et al., 2005), which generates V2a and V2b interneurons. We analyzed

(Fig. 3D, E and not shown). Conversely, *Foxn4* was expressed as normal in *Scl* conditional null mice (2/2 embryos) (Fig. 3F, G). Also, as described above, *Foxn4* induces *Scl* expression after 48h (17/17 embryos) (Fig.3C). Therefore, it seems that *Foxn4* lies upstream of *Scl* in the genetic hierarchy leading to V2b INs.

In control experiments we electroporated a vector identical to *β-actin-Foxn4*, except that the *Foxn4* coding sequence between the poly-linkers was inverted. In none of the eight embryos analyzed did we find ectopic expression of *Gata2, Gata3* or *Scl* (not shown). Taken together, our data suggest that *Foxn4* might be a master regulator of the V2b lineage. Further, we have shown that Scl lies downstream of Foxn4 in the pathway that governs development of V2b INs.

Ectopic Foxn4 represses interneuron fates outside the p2 domain

Foxn4 induces ectopic expression of V2b markers in the dorsal spinal cord (Fig. 2), raising the possibility that it might be a master regulator of the V2b sub-lineage. To test this further we asked whether Foxn4 can repress alternative fates in the dorsal cord. We electroporated *β-actin-Foxn4-IRES-GFP* into the embryonic chick neural tube at st14 and immunolabelled sections 24 hours later with anti-Engailed1 (En1), which labels postmitotic V1 interneurons (Ericson et al., 1997) and anti-Lhx1/2, which labels postmitotic interneurons derived from progenitor domains dP1-dP6 with the exception of dP3 domain (reviewed by Lewis et al., 2006). Foxn4 was able to repress both of these markers (Fig. 4). A reduction of 31% ± 3% (mean ± standard error, n=4) was observed for En1 (1,173 cells on the control side versus 776 on the electroporated side, 39 sections from four embryos) (Fig. 4A) and a reduction of 45% ± 13% (n=4) for Lhx1/2 (4,239 cells on the control side versus 2,604 on the electroporated side, 30 sections from four embryos) (Fig. 4B). The negative control vector with inverted *Foxn4* sequences had no activity (not shown). Together with the results described in the previous paragraph, this suggests that ectopic expression of Foxn4 reprograms progenitors to a V2b IN fate.

Foxn4 is expressed in the common progenitors of V2a and V2b INs

It was previously reported that V2a and V2b INs share common, Foxn4-expressing progenitor cells in the VZ (Li et al., 2005). We confirmed this by following expression of β-galactosidase in *Foxn4* (+/-) heterozygotes – which is possible because the knockout allele contains a functional copy of *LacZ* under *Foxn4* transcriptional control. By double immunohistochemistry we found that β-galactosidase protein was present in cells that co-express Chx10 (Fig. 5A) as well as in cells that express Gata3 (Fig. 5B). In contrast, *Foxn4* transcripts or protein were never found in the same cells as Chx10 or Gata3 (Fig. 1H) (Li et al., 2005). The most parsimonious interpretation is that there is a common pool of Foxn4-positive progenitors that generates both V2a and V2b INs. The

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ectopic expression of Chx10 protein or mRNA but a strong repression of Chx10 protein on the electroporated versus control side (51% ± 5 % reduction, mean ± standard error. 137 sections from 13 embryos, two-tail t-test=3.6 at p=0.001) (Fig. 7A, B). In these experiments *Gata2* mRNA was expressed ectopically in some embryos (19/63 sections in 5 out of 15 embryos). In general, the induction of *Gata2* was modest and always restricted to the p1-p0 domain (Fig. 7D', white arrow). Despite this small amount of ectopic expression the total amount of *Gata2* signal (estimated by counting pixels with the Image-J program) was not detectably different on the electroporated versus control sides (594 ± 83 versus 562 ± 83 pixels respectively, 80 sections from 7 embryos, two-tail t-test=0.3 at p=0.8, not significant) (Fig. 7D',E). The *Scl* signal was also not significantly different between electroporated and control sides (396 ± 64 pixels versus 361 ± 57 respectively, 86 sections from 9 embryos, two-tail t-test=0.5 at p=0.6, not significant) - nor was there any ectopic expression of *Scl* (Fig. 7D'', E). These results suggest that at st14-16 Dll4 over-expression specifically represses the V2a fate with little or no effect on V2b fate.

It is perhaps significant that in *Dll4* electroporations some cells were Dll4-Myc/ Chx10 double-positive (Fig. 7C), indicating that expression of Dll4 is compatible with expression of Chx10 in the same cell. This is consistent with the notion that the inhibition of Chx10-positive V2a INs that we observe is through the action of Dll4 on neighbouring cells (i.e. a non cell-autonomous activity of Dll4) - as expected for conventional Delta-Notch signalling.

Foxn4 induces Mash-1/Ascl1 in the p2 domain

The extensive overlap of *Mash-1* and *Foxn4* expression in the mouse p2 domain (Figs. 5C, 8A) suggested some form of regulatory relationship. We therefore explored the interactions between *Foxn4* and *Mash-1* in more detail. We confirmed the finding of Li et al. (2005) that *Foxn4* is expressed as normal in *Mash-1* null spinal cord (Fig. 8C, D). After electroporating *β* Tc

Mash-1 by ISH and immunohistochemistry for GFP (Fig. 8B, E, F, H and not shown). After 24 hours of incubation 8/8 embryos showed clear ectopic induction of *Dll4* on the electroporated side (Fig. 8E). After 48 hours 5/5 embryos displayed weaker but still clear induction of *Dll4* (not shown). In none of the thirteen embryos analyzed did we find any ectopic expression of *Chx10, Gata2* or *Scl* transcripts or Chx10 immunoreactivity (not shown, Fig. 8H', H'', F, G respectively). On the other hand, we found a loss of endogenous Chx10-positive INs in the p2 domain of 5/5 embryos analyzed (76 ± 6% reduction, n = 23) (Fig. 8F, F', G), with little or no concomitant reduction of *Gata2* or *Scl* (Fig. 8H', H''). These data suggested that induction of *Dll4* and consequent repression of Chx10-positive V2a INs by Foxn4 might be mediated indirectly via Mash-1/Ascl1. However, we have found that *Dll4* is expressed as normal at E10.5-11 in *Mash-1* null embryos (4/4 embryos; data not shown). Therefore, (ll4pD0 TcM(sh- (m)-19.2(i)-2.3(ght (e)17.8t (volved)17.9((

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more consistent with re-specification of V2b to V2a INs, consistent with the idea that signalling through Notch-1 is required for V2b IN development.

Foxn4 is very much reduced in the E11.5 *Notch-1* conditional null spinal cord (Fig. 9I, J, arrow). A

Gata3 expression (Karunaratne et al., 2002;Nardelli et al., 1999) and suggests that *Gata2* is genetically upstream of *Scl*. This is backed up by the fact that *Gata2* is expressed ahead of *Scl* during normal development in both chicks and mice (Muroyama et al., 2005 and data not shown). *Gata3* expression is lost in *Scl* null mice, placing *Scl* upstream of *Gata3* (Muroyama et al., 2005). Taken together, the available data support a genetic cascade *Foxn4 -> Gata2 -> Scl -> Gata3*. The reduction of *Gata2* expression that was observed in *Scl* null mice (Muroyama et al., 2005) can be attributed to loss of positive feedback from Gata3 (Karunaratne et al., 2002). A diagram of the proposed network is shown in Fig. 10.

Foxn4 activates Dll4 and Mash-1/Ascl

By loss- and gain-of-function experiments we found that *Foxn4* is necessary and sufficient to activate *Dll4* and *Mash-1* expression. We subsequently showed that Mash-1 also can induce ectopic expression of *Dll4* in chick spinal cord. This suggests that the conserved Mash-1/Brn binding site in the *Dll4* upstream region, recently reported by Castro et al. (2006), is functional in vivo and further suggested that Foxn4 might activate *Dll4* indirectly through Mash-1. However, we found that Mash-1 is not required for initiation of *Dll4* expression in the mouse because *Dll4* is expressed normally in the p2 domain of E10.5 *Mash-1* null spinal cord. It is possible that Mash-1 might be required to maintain *Dll4* expression after E10.5 but we have not examined older embryos. Alternatively, a requirement for Mash-1 in the initiation of *Dll4* expression might be masked in *Mash-1* mutant mice through compensatory up-regulation of a related proneural factor such as Ngn1 or Ngn2. It is also possible that Foxn4 induces *Dll4* directly; in endothelial cells, for example, Foxc1 and/or Foxc2 are known to activate *Dll4* by binding directly to a Fox binding site in the *Dll4* gene upstream region (Seo et al., 2006).

Apart from regulating *Dll4*, Mash-1 must have another role in promoting V2b IN fate, because *Mash-1* null mice at E10.5 are reported to have ~50% less V2b INs than normal (Li et al. 2005), despite the fact that *Foxn4* and *Dll4* are both expressed normally (Fig. 8D and not shown), More work needs to be done to establish the precise role of Mash-1 in V2b IN development.

Notch-1 is required for V2b interneuron development

The connection between Foxn4, Dll4 and Mash-1/Ascl1 led us to explore the role of Delta-Notch signalling more directly. We previously reported that when Notch-1 function was disrupted in the ventral spinal cord by expression of a *Nestin-Cre* transgene in floxed *Notch-1* mice, the result was a ~30% overproduction of (Chx10, Lhx3) double-positive V2a INs and an ~18% loss of (Islet1, Lhx3) double-positive MNs (Yang et al., 2006). This was originally interpreted as a fate switch

Dll4/Notch-1 signalling breaks symmetry and splits the V2 lineage

We followed the fates of *Foxn4*-expressing progenitors directly in heterozygous *Foxn4 (+/-)* mice, which express *LacZ* under *Foxn4* transcriptional control. The encoded β-gal protein was found in both V2a and V2b INs, demonstrating that both V2 subtypes descend from *Foxn4*-positive progenitors. In addition, *Foxn4* is co-expressed with markers of both V2a and V2b INs (*Gata2, Lhx3, Mash-1*) at the ventricular surface, where progenitor cell mitosis occurs. It therefore seems probable that there is a population of bipotential, Foxn4-positive V2 progenitors that generates V2a and V2b INs simultaneously under the action of Notch-Delta.

What is the mode of action of Notch-1 in V2 interneuron development? One possibility might be that p2 progenitors normally generate V2a INs first, before switching to V2b production, and that Dll4/Notch-1 is needed to keep some progenitors in cycle long enough to generate V2b INs. In that case, abrogation of Notch signalling might be expected to cause accelerated differentiation along the V2a pathway and loss of V2b differentiation, as observed. However, there is no evidence that V2a INs are formed before V2b INs. *Chx10* and *Gata3* are both expressed together for the first time at E10.5 in mouse (Liu et al.,

division(s) before terminal differentiation. Either of these scenarios would be consistent with our observations that approximately equal numbers of V2a and V2b INs are formed under normal circumstances and that twice the normal number of V2a INs form in the absence of Notch-1 (Fig. 9D).

Note that our proposed roles for Mash-1 and Dll4/Notch-1 signalling in separating V2a and V2b lineages is closely analogous to the roles proposed for Mash-1 and Dll1/Notch in specifying

Electroporation of chick embryos in ovo

Fertilised chicken eggs were incubated at 38°C in a humidified incubator, opened and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were electroporated at st11-16 (Itasaki et al., 1999). The expression constructs [2-5 µg/µl in phosphate-buffered saline (PBS) and 0.8% (w/v) Fast Green] were injected into the lumen of the spinal cord and electroporated using an Intracel TSS20 Ovodyne electroporator with EP21 current amplifier and 0.5 mm diameter home-made platinum electrodes (4-5 pulses of 20-25 volts for 50 ms each).

Tissue preparation and immunohistochemistry

Embryos were dissected in cold PBS and fixed in 4% (w/v) paraformaldehyde in PBS. They were then cryo-protected with 20% (w/v) sucrose in PBS, embedded in OCT and frozen for cryo-sectioning (10 µm nominal thickness). The antibodies used were: rabbit polyclonal anti-GFP at 1:8000 (#ab290-50, Abcam), rabbit anti-Chx10 at 1:100 (provided by Thomas Jessell and Connie Cepko), mouse monoclonal anti-Myc at 1:200 (#M4439, Sigma), mouse monoclonal anti-Gata3 at 1:100 (#SC268, Santa Cruz), rabbit anti-Olig2 1:8000 (provided by Charles Stiles), mouse monoclonal anti-Hb9 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-gal at 1:2000 (Cappel, ICN Pharmaceuticals), mouse anti-Lhx2 at 1:30 (DSHB), mouse anti-En1 at 1:5 (DSHB), mouse anti-β-gal (Promega) at 1:300 (with tyramide amplification, Molecular Probes). Some of the sections were incubated with DAPI in PBS in order to visualize cell nuclei before mounting.

In situ hybridization

Our ISH protocols are as described (http://www.ucl.ac.uk/~ucbzwdr/richardson.htm

templates: Luis Puelles for chick *Nkx6.1*, Graham Goodwin for chick *Scl*, Thomas Reh for *Cash-1*, Henrique Domingos for mouse *Dll4*, Janette Nardell for mouse *Chx10* and Stuart Orkin for mouse *Gata2*. Raquel Taveira-Marques is supported by a studentship from the Portuguese Fundação para a Ciência e a Tecnologia. This work was supported by grants from the US National Institutes of Health [R01 NS047572 (DHR) and R01 NS042818 (JS)], the Wellcome Trust and the UK Medical Research Council (WDR).

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visualized by ISH at E11.5 in wild type (F) and *Scl* conditional null mouse spinal cords (G) (see Methods). Taken together, these data demonstrate that *Foxn4* is genetically upstream of *Scl*.

Figure 4 Ectopic Foxn4 expression represses interneuron fates other than V2a. Chick embryos were electroporated at st14 with *β-actin-Foxn4-IRES-GFP* and analyzed after 24 hours by double immunolabelling with anti-GFP (green) and either anti-En1 or anti-Lhx1/2 (red). The numbers of both En1-positive (A) and Lhx1/2-positive (B) cells was reduced (see text for quantification).

Figure 5 *Foxn4* is expressed in common precursors of V2a and V2b INs. (A, B) *Foxn4 (+/-)* embryos were labelled by double immunohistochemistry for β-galactosidase (β-gal, green) and either Chx10 or Gata3 (red) (see text for details). Confocal microscopy reveals cells that are double-labelled for β-gal and either Chx10 (A) or Gata3 (B), suggesting that Foxn4-expressing progenitors give rise to both V2a and V2b INs. Consistent with this conclusion, *Foxn4*-positive progenitors co-express *Mash-1* (C) and *Lhx3* (D), markers that later segregate into V2b and V2a INs respectively.

Figure 6 *Foxn4* is necessary and sufficient to induce *Dll4* in the p2 domain. (A-C) Double ISH for *Dll4* (green)-*Foxn4* (red) in wild type E10.5 mouse embryos, counter-stained with Hoechst stain to visualize cell nuclei. (A) is a transverse section of spinal cord and (B) a longitudinal section. *Foxn4* is expressed in some of the *Dll4*-positive cells within and outside the VZ (arrows). A

Figure 8 Foxn4 controls *Mash*-1 expression. (A) Double ISH for *Foxn4* (red) and *Mash-1* (green)

and that Gata3 is abolished and Gata2 severely reduced in *Scl* null mice; Li et al. (2005) showed that *Mash-1* expression is abolished in *Foxn4* null mice.

Figure 11 Generation of V2a and V2b INs from common progenitors in the p2 domain. Multipotent neuroepithelial (radial) progenitors (A), which do not express Foxn4, generate a population of V2a/V2b (p2) progenitors (B). All V2a/V2b progenitors express Foxn4, which induces the expression of Dll4, Gata2 and Mash-1. These common progenitors also start to express Lhx3 at their final division (C). Notch-1 is expressed in all p2 progenitors (Lindsell et al., 1996), so Notch-1/ Dll4 reciprocal cell-cell interactions are initiated (opposing arrows in C). This situation resolves into two populations of progenitors, one with activated Notch-1 (Notch-1*) and the other with Dll4 (D). Notch-1* blocks the V2a fate and, in cooperation with Foxn4 and Mash-1, specifies V2b IN fate (E). The complementary set of p2 progenitors (Dll4-positive) that fails to activate Notch-1 adopts the V2a fate instead, possibly under the control of Lhx3 (Tanabe et al., 1998) (E). In this way V2a and V2b interneurons are generated in salt-and-pepper fashion during the same time window from a homogeneous population of p2 progenitors.

Foxn4

Foxn4

Foxn4


A
A'
B
B'
Foxn4
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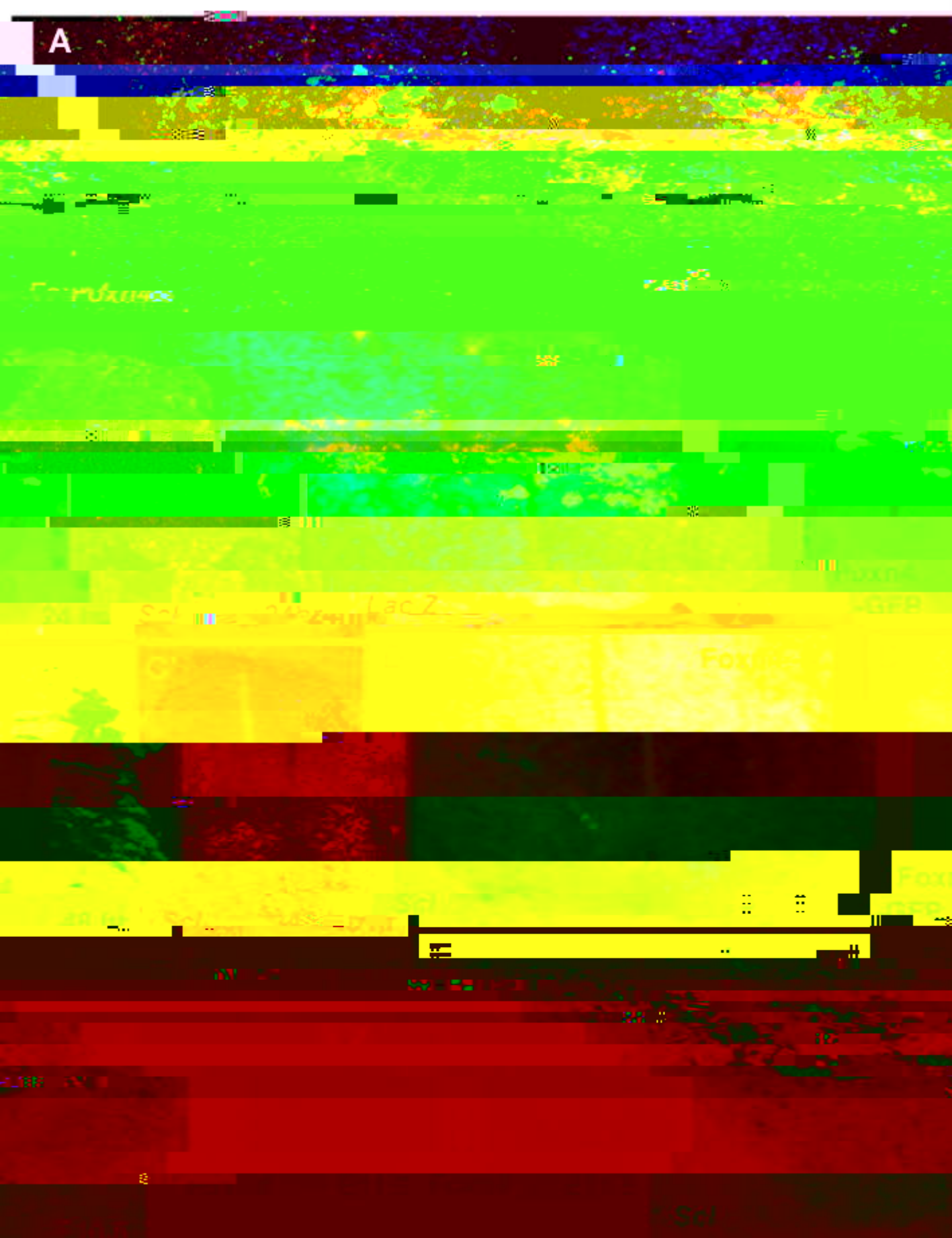
Gata2
24 hr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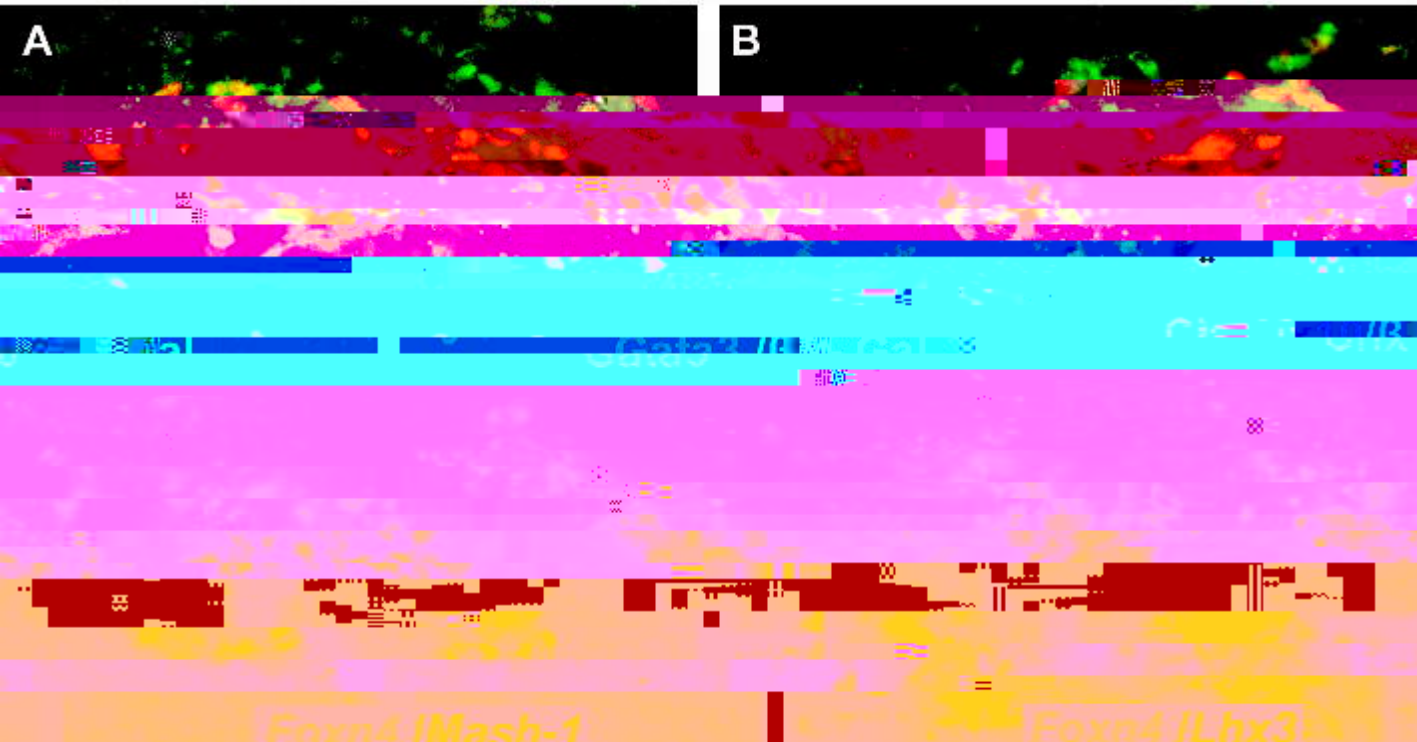
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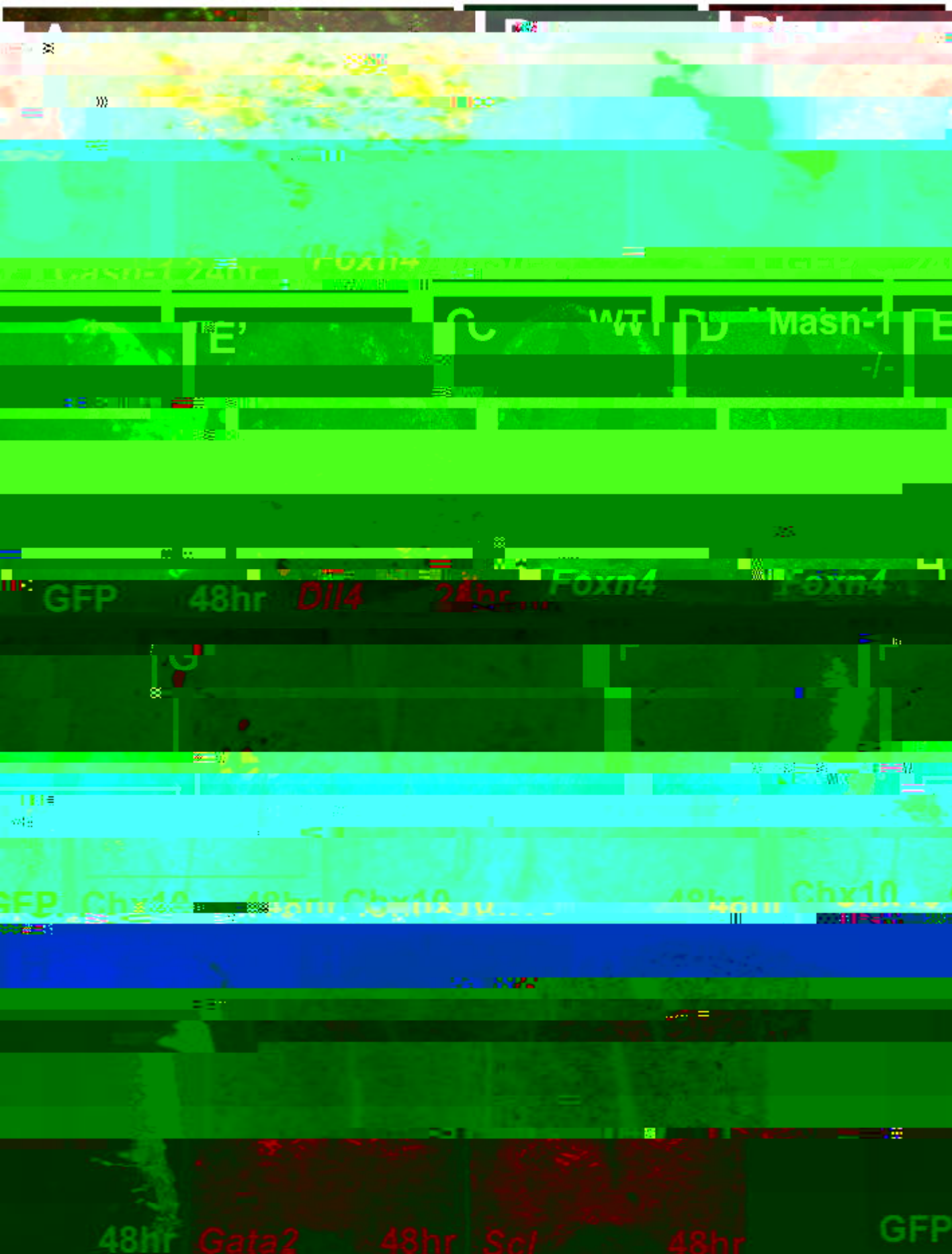
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Scl
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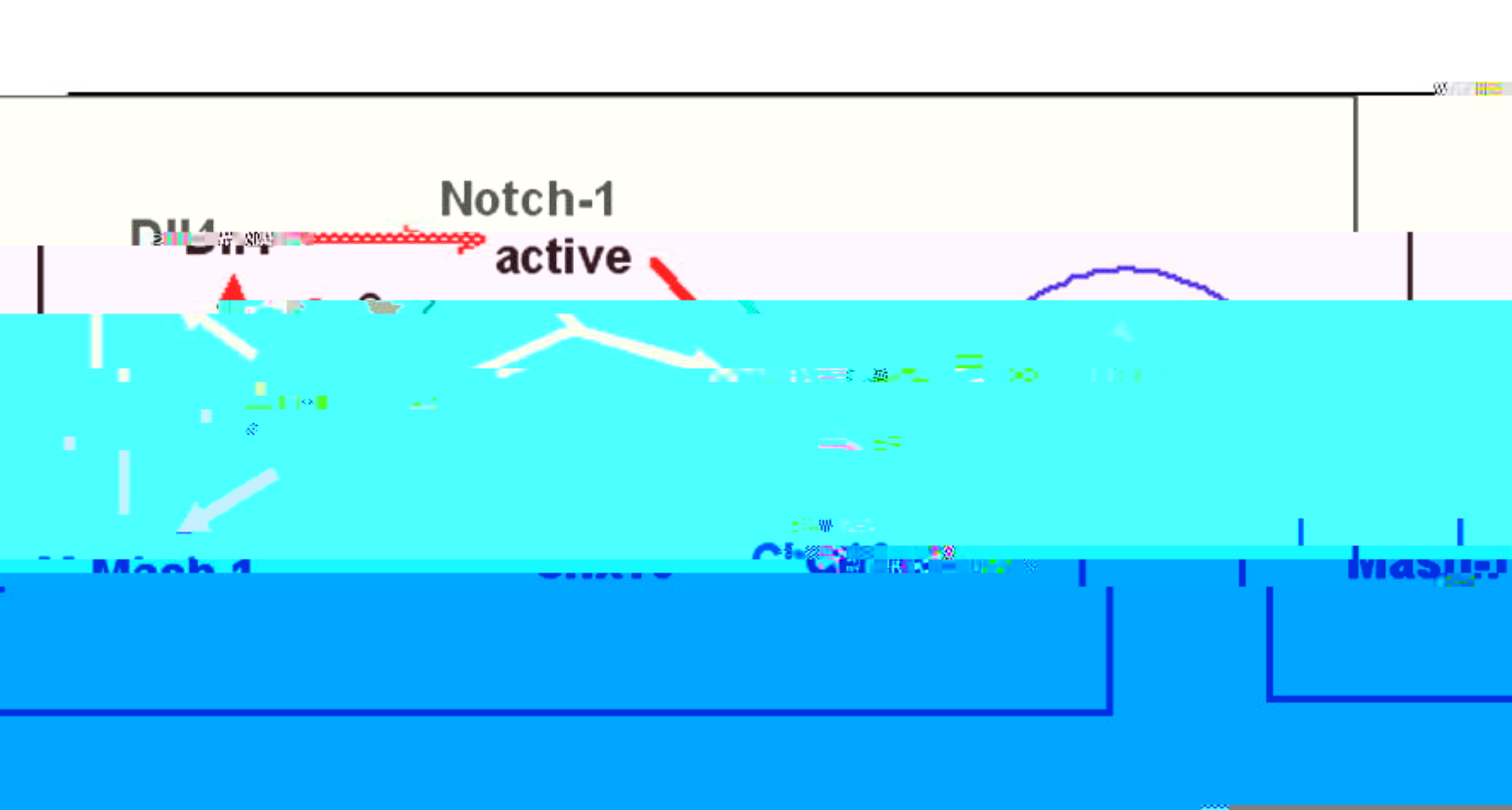
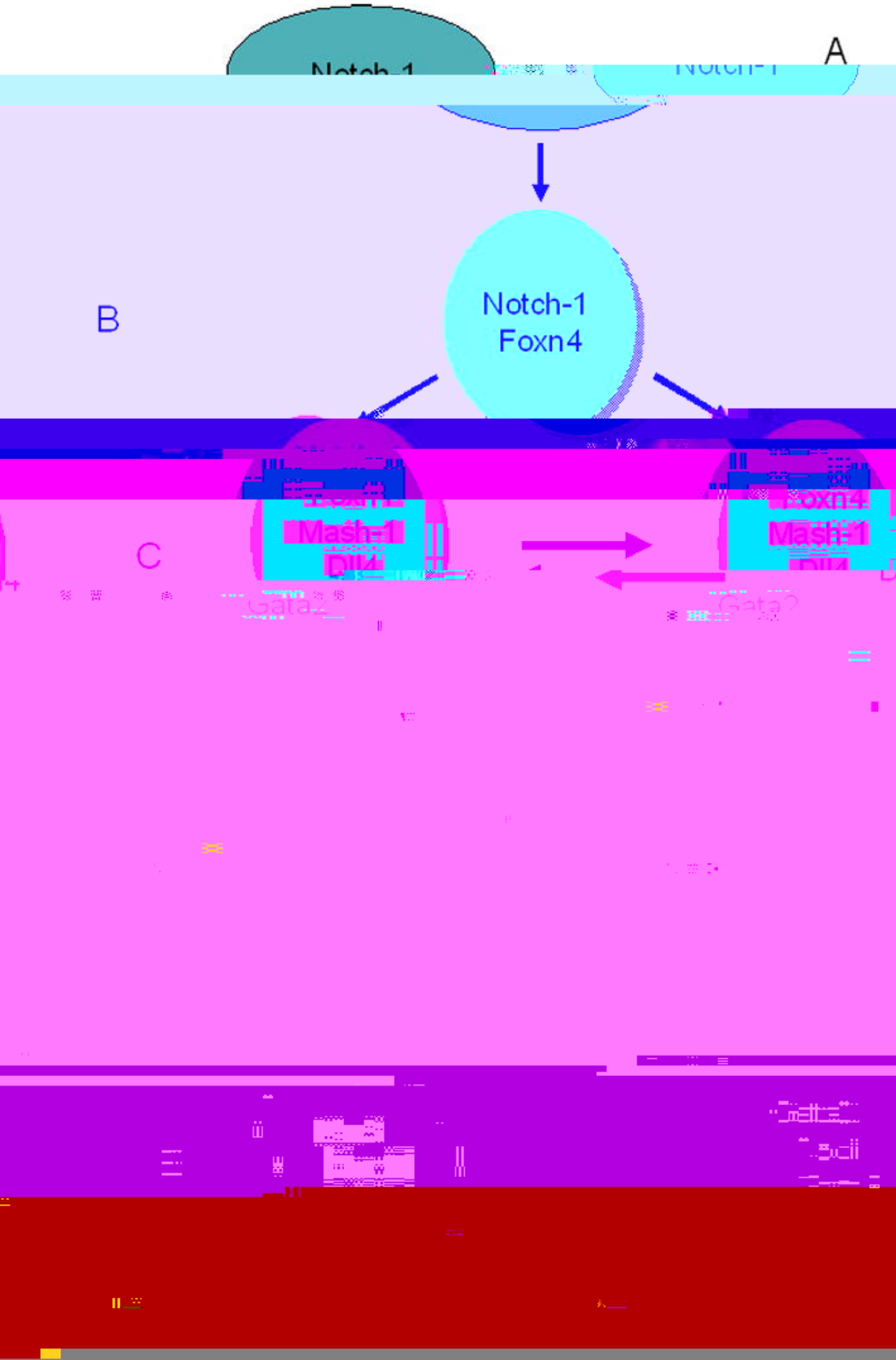

Notch-1
active


A
Notch-1
B
Notch-1
Foxn4
C
Mash-1
Foxn4
Mash-1