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RESEARCH ARTICLE

The PAX-SIX-EYA-DACH network modulates GATA-FOG function in fly hematopoiesis and human erythropoiesis

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ABSTRACT

The GATA and PAX-SIX-EYA-DACH transcriptional networks (PSEDNs) are essential for proper development across taxa. Here, we demonstrate novel PSEDN roles *in vivo* in *Drosophila* hematopoiesis and in human erythropoiesis *in vitro*. Using *Drosophila* genetics, we show that PSEDN members function with GATA to block lamellocyte differentiation and maintain the prohemocyte pool. Overexpression of human SIX1 stimulated erythroid differentiation of human erythroleukemia TF1 cells and primary hematopoietic stem-progenitor cells. Conversely, SIX1 knockout impaired erythropoiesis in both cell types. SIX1 stimulation of erythropoiesis required GATA1, as SIX1 overexpression failed to drive erythroid phenotypes and gene expression patterns in GATA1 knockout cells. SIX1 can associate with GATA1 and stimulate GATA1-mediated gene transcription, suggesting that SIX1-GATA1 physical interactions contribute to the observed functional interactions. In addition, both fly and human SIX proteins regulated GATA protein levels. Collectively, our findings demonstrate that SIX proteins enhance GATA function at multiple levels, and reveal evolutionarily conserved cooperation between the GATA and PSEDN networks that may regulate developmental processes beyond hematopoiesis.

KEY WORDS: GATA, SIX1, SIX2, Retinal determination gene network, PAX-SIX-EYA-DACH network, Hematopoiesis

INTRODUCTION

GATA transcription factors orchestrate the development of diverse tissues across taxa and act at multiple stages during tissue development, including stem-progenitor cell genesis, maintenance, differentiation and maturation (Aronson et al., 2014; Lentjes et al., 2016; Patient and McGhee, 2002). Dysregulation of these processes leads to developmental disorders and malignancies that reflect distinct GATA factor expression patterns and tissue-specific functions (Chou et al., 2010; Crispino and Horwitz, 2017; Gao et al., 2015; Katsumura et al., 2017, 2016; Lentjes et al., 2016; Shimizu and Yamamoto, 2016; Zheng and Blobel, 2010). Humans possess six GATA family members, three of which function in hematopoiesis (Katsumura et al., 2017). GATA1 is a master regulator of erythropoiesis from zebrafish to humans (Lyons et al., 2002; Nichols et al., 2000; Pevny et al., 1991; Rekhtman et al., 1999), and is also required for mammalian megakaryocyte (Shivdasani et al., 1997; Stachura et al., 2006; Vyas et al., 1999), mast cell (Migliaccio et al., 2003), basophil (Nei et al., 2013) and eosinophil (Yu et al., 2002) differentiation. *Gata1* knockout mice die embryonically due to severe anemia (Fujiwara et al., 1996), consistent with essential roles of Gata1 in erythroid progenitor specification, viability, proliferation and terminal differentiation (Gregory et al., 1999; Mancini et al., 2012; Pan et al., 2005; Rylski et al., 2003; Weiss and Orkin, 1995). GATA2 is essential for formation and maintenance of hematopoietic stem-progenitor cell populations (Ling et al., 2004; Tsai and Orkin, 1997). GATA3 is required for lymphocyte development and is involved in regulation of hematopoietic stem cells (Fitch et al., 2012; Frelin et al., 2013; Ku et al., 2012; Zaidan and Ottersbach, 2018). Both *Gata2* and *Gata3* knockout mice die embryonically due to disruption of definitive hematopoiesis (Pandolfi et al., 1995; Tsai et al., 1994).

GATA factor output is modulated by specific co-factor interactions, which contribute to complex developmental and cell type-specific diversity of GATA function. For example, evolutionarily conserved Friend of GATA (FOG) proteins are zinc-finger proteins that bind the N-terminal zinc finger of their respective GATA-binding partners to regulate GATA function. FOG proteins do not bind DNA directly, but shape GATA output via modulation of GATA chromatin occupancy (Chlon et al., 2012) and recruitment of additional co-factors, such as PIASy E3 ligase or the NuRD complex, that mediate GATA-directed gene activation or repression, respectively (Hong et al., 2005; Lee et al., 2009; Miccio et al., 2010). Humans possess two FOG family members, one of which, FOG1 (also known as ZFPM1), promotes GATA1-mediated erythroid and megakaryocytic differentiation but opposes the differentiation of eosinophils, mast cells and granulocytes (Cantor et al., 2008; Chang et al., 2002; Gao et al., 2010; Querfurth et al., 2000; Tsang et al., 1998, 1997). Like mice lacking *Gata1*, *Fog1* knockout mice die early in embryogenesis due to severe anemia (Tsang et al., 1997), and in humans an inherited mutation in *GATA1* abrogating GATA1-FOG1 interaction produces familial dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000). Additional GATA co-factors, including HSP70 (Ribeil et al., 2007; Rio et al., 2019), P53 (Trainor et al., 2009), and LMO2 (Wadman et al., 1997;

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Wilkinson-White et al., 2011), impact GATA function by regulating GATA protein stability and transcriptional output. Elucidation of GATA co-regulators is central to dissecting the complex roles GATA family members play during development.

Drosophila melanogaster provides a powerful model system for investigating GATA function *in vivo*, enabling the identification of conserved co-regulators derived from ancient core hematopoietic networks (Fossett, 2013). The comparatively simple *Drosophila* blood system shares important commonalities with vertebrate hematopoietic systems, including distinct spatial and temporal regulation, and multipotent progenitors (prohemocytes) that give rise to all three mature blood cell types. These blood lineages (plasmatocytes, crystal cells and lamellocytes) carry out functions associated with the vertebrate myeloid lineages (Dearolf, 1998; Lanot et al., 2001). Plasmatocytes are operational macrophages; crystal cells are involved in wound healing; and lamellocytes, which are rarely observed under steady-state conditions, differentiate to combat wasp parasitization and in response to other forms of stress, including increased levels of reactive oxygen species (ROS) and nutritional deprivation (Frandsen et al., 2008; Gold and Brückner, 2015; Owusu-Ansah and Banerjee, 2009; Rizki and Rizki, 1992; Shim et al., 2012; Sorrentino et al., 2002; Vlisidou and Wood, 2015).

The *Drosophila* hematopoietic GATA factor Serpent (Srp) exerts functions analogous to all three vertebrate hematopoietic GATAs, ranging from prohemocyte specification to differentiation of blood lineages (Mandal et al., 2004). Like mammalian GATAs, Srp interacts with a variety of conserved hematopoietic transcriptional regulators (Ush/Fog, Runx, NuRD complex) and regulatory pathways (BMP, Hh, JAK/STAT) that direct specific hematopoietic Srp/GATA activities, including cell fate and differentiation (Baldeosingh et al., 2018; Fossett, 2013; Frandsen et al., 2008; Gao et al., 2009; Muratoglu et al., 2006; Tokusumi et al., 2010; Waltzer et al., 2002) (J. Lenz, R. Liefke, J. S. Funk, A. Nist, T. Stiewe, R. Schulz, Y. Tokusumi, L. Alberts, K. Förstemann, O. Vazquez, T. Tokusumi, N.G.F. and A. Brehm, unpublished observations). In prohemocytes, the *Drosophila* FOG protein U-shaped (Ush) binds to GATA to block lamellocyte differentiation and thereby maintain the prohemocyte population (Mandal et al., 2004). We previously conducted a second-site non-complementation (SSNC) screen in *Drosophila* to identify novel conserved *in vivo* GATA network members based on the role of GATA in lamellocyte differentiation (Baldeosingh et al., 2018). SSNC refers to the occurrence of a mutant phenotype in double heterozygotes harboring heterozygous recessive mutations in distinct genes. SSNC occurs when the functional output of cellular pathways drops below the threshold required to maintain the wild-type phenotype, thereby enabling SSNC screens to identify proximal or distal upstream regulators, co-regulators, downstream effectors and parallel pathways (Hawley and Gilliland, 2006).

Here, we show that *Drosophila* PAX-SIX-EYA-DACH transcriptional network (PSEDN) members *eyeless* (*ey/PAX*), *sine oculis* (*so/SIX*), *eyes absent* (*eya/EYA*) and *dachshund* (*dac/DACH*) genetically interact with *ush*/*FOG* to block lamellocyte differentiation. So and Eya support the ability of Srp:Ush to block lamellocytes differentiation and maintain the prohemocyte pool. We further show that two human homologs of So, SIX1 and SIX2, enhance GATA1 function and stimulate erythropoiesis in a human erythroleukemia cell line and primary hematopoietic stem-progenitor cells (HSPCs). In contrast, SIX1 knockout impaired erythropoietin (EPO)-stimulated erythropoiesis. The PSEDN (Davis and Rebay, 2017; Tadjuidje and Hegde, 2013) directs the development of diverse mammalian organ systems (Davis and Rebay, 2017; Li et al., 2003; Tadjuidje and Hegde, 2013; Xu, 2013). Loss of EYA1 or SIX1 function leads to branchio-oto-renal (BOR) syndrome (Abdelhak et al., 1997; Kochhar et al., 2007), whereas gain of function has been associated with diverse cancers, including leukemias (Blevins et al., 2015; Chu et al., 2019; Kingsbury et al., 2019; Kong et al., 2016; Liu et al., 2016; Wang et al., 2011; Zhang et al., 2018). Our findings thus expand the repertoire of GATA1 hematopoietic co-regulators by revealing previously unknown evolutionarily conserved functional interactions between two major developmental transcriptional networks, the GATA network and PSEDN, each with crucial roles in normal and malignant development.

RESULTS

Genetic interactions between FOG and PSEDN block lamellocyte differentiation in fly hematopoiesis

Like vertebrates, *Drosophila* hematopoiesis takes place during spatially and temporally distinct periods or waves. The first wave takes place in the embryonic head mesoderm, whereas the second wave takes place during the larval stage in a specialized organ known as the lymph gland (Fossett and Schulz, 2001). Blood cells from both waves persist throughout the adult stage of the fly (Ghosh et al., 2015). During the larval stage, blood cells from the first wave make up the population of circulating cells (Holz et al., 2003). In *Drosophila*, Ush/FOG acts with Srp/GATA to block lamellocyte differentiation and maintain prohemocytes (Gao et al., 2013). The loss of Ush/FOG reduces the prohemocyte population and dramatically increases lamellocyte differentiation (Gao et al., 2009; Sorrentino et al., 2007). The resulting increase in circulating lamellocytes provides a robust phenotype that we exploited to identify a novel GATA network (Baldeosingh et al., 2018).

Taking advantage of the requirement for Ush to block Srp-mediated lamellocyte differentiation, we conducted SSNC using a fly background harboring a heterozygous null allele of *ush* (*ush*^{vx22}) marked by *misshapen-mCherry* (*MSN-C*) fluorescent reporter gene on the same chromosome (Baldeosingh et al., 2018). *MSN-C* is a marker for lamellocytes (Tokusumi et al., 2009), which allowed us to rapidly identify larvae with increased numbers of lamellocytes using fluorescence microscopy. SSNC screening was performed using *ush* heterozygotes because Srp is required for all stages of hematopoiesis, including prohemoctye formation, and perturbation of Srp expression levels produces pleiotropic effects that confound functional analyses. Screening chromosomal deficiencies spanning the entirety of chromosome 2L (which makes up ∼20% of the fly genome) identified a deficiency, *Df(2L)BSC354*, containing the gene encoding the PSEDN member *eyes absent* (*eya*). Genetic interaction between *eya* and *ush* was confirmed by crossing *ush* single heterozygous flies harboring the mCherry lamellocyte marker (*ush*^{vx22} *MSN-C/CyO*) to *eya* single heterogygous flies (*eya*$^{cli-IID}$ *cn*^{1} *bw*^{1} *sp*^{1}*/CyO*). Larval populations containing *ush/eya* double heterozygotes displayed a dramatic increase in circulating lamellocyte numbers compared with either *ush/+* or *eya/+* singularly heterozygous populations, which resembled wild-type controls (Table 1). Consistent with the expected 50% frequency of *eya/ush* double heterozygotes, approximately half (16/29) of the *ush* heterozygous larvae exhibited a >10-fold increase in circulating lamellocytes compared with wild-type controls (1/67) or singularly heterozygous *ush/+* (2/30) or *eya/+* (0/22) larval populations. Similar results were observed in the larval hematopoietic lymph gland (Fig. S1). Both *ush/+* and *eya/+* single heterozygote larvae exhibited very few lamellocytes within the lymph gland, comparable with wild-type controls. In contrast, *ush/eya* double heterozygotes displayed a dramatic increase in lamellocytes, suggesting *eya* genetically interacts with *ush* to block lamellocyte differentiation.

Development (2020) 147, dev177022. doi:10.1242/dev.177022

Table 1. PAX-SIX-EYA-DACH transcriptional network members exhibit second-site non-complementation with *ush* (FOG)

PSEDN member	*Drosophila* allele	*ush*/+ PSEDN member/+ (trans-heterozygotes)		*PSEDN* member/+ (PSEDN single heterozygotes)		*P*-value‡
		Number of positives*/ number scored	% Positive	Number of positives*/ number scored	% Positive	
NA	Control	2/30	6.7	1/67	1.6	0.2252§
EYA	$eya^{Cli\text{-}IID}$/+¶	16/29	55	0/22	0	>0.0001
SIX	so^{3}/+¶	18/20	90	7/61	11.5	>0.0001
	so^{+2}/+¶	7/34	21	2/51	4	>0.02
	so^{1}/so^{1}**	11/29	38	1/45	2.2	>0.0001
PAX	ey^{2}/ey^{2}**	16/16	100	11/47	23.4	>0.0001
	ey^{1}/ey^{1}**	37/38	97			NA
DACH	dac^{1}/+¶	11/21	52	8/70	11.4	>0.0003
	dac^{9}/+¶	15/21	71	6/82	7.3	>0.0001
	dac^{e462}/+¶	13/20	65	4/73	5.5	>0.0001

*Estimated ≥10× increase in lamellocyte number.

‡Fisher's exact test for increased percentage of lamellocytes in trans-heterozygotes versus single heterozygotes.

§LMS observed in *ush* heterozygotes versus wild-type controls.

¶PSEDN heterozygotes crossed to *ush MSN-C*/+; 50% of scored (*MSN-C* progeny) expected to be trans-heterozygotes.

**PSEDN homozygotes crossed to *ush MSN-C*/+; 100% of scored (*MSN-C* progeny) expected to be trans-heterozygotes.

To determine whether additional PSEDN members genetically interact with GATA-FOG, *ey*, *so* and *dac*, were tested for SSNC with *ush*. When carried as double heterozygotes with *ush*, each PSEDN member exhibited increased lamellocyte numbers compared with larval populations singularly heterozygous for either *ush* or a given PSEDN member (Table 1). These observations demonstrate that the PSEDN genetically interacts with GATA-FOG during *Drosophila* hematopoiesis. Several PSEDN alleles also exhibited elevated lamellocyte numbers as single heterozygotes (Table 1), revealing a previously unappreciated role for the PSEDN in *Drosophila* hematopoiesis. Given the conservation of Eya-So/EYA-SIX physical and functional interactions across species, we focused our analyses on these two PSEDN members.

Eya and So function in prohemocytes to block lamellocyte differentiation and to maintain prohemocyte number

In the larval lymph gland, multipotent prohemocytes reside in the inner region of the primary lobe of the lymph gland, known as the medullary zone (MZ), while differentiating blood cells reside in the cortical zone (CZ) (Jung et al., 2005; Krzemień et al., 2010; Mandal et al., 2007). Multipotent prohemocytes can be readily identified and genetically manipulated in the larval hematopoietic lymph gland (Jung et al., 2005; Krzemień et al., 2010; Mandal et al., 2007). Previously, we reported that *Domeless-Gal4* (*Dome-Gal4*)-driven RNAi knockdown of Ush in prohemocytes increased lymph gland lamellocyte differentiation (Gao et al., 2013). Likewise, we observed that *Dome-Gal4*-driven RNAi knockdown of Eya phenocopied Ush knockdown; substantially increasing lamellocyte differentiation (Fig. 1A-C). Thus, Eya functions in prohemocytes to block lamellocyte differentiation.

Increased lamellocyte differentiation is generally associated with prohemocyte loss (Gao et al., 2009; Krzemień et al., 2007). We therefore tested whether Eya knockdown reduced prohemocyte numbers. The transcription factor Odd-skipped (Odd), which maintains E-cadherin-positive prohemocytes, was used as a marker for prohemocytes (Baldeosingh et al., 2018; Gao et al., 2011). As reported for Ush (Baldeosingh et al., 2018), knockdown of Eya in prohemocytes resulted in a significant reduction in the Odd-positive expression domain (Fig. 1D-F), indicating that Eya is required to maintain Odd-positive prohemocytes.

As Eya functions as a co-activator for the transcription factor So (Davis and Rebay, 2017), we further tested the consequences of So knockdown in prohemocytes. So knockdown using either the *Dome-Gal4* (Fig. S2) or a second prohemocyte-restricted driver *Tep-Gal4* reduced Odd-positive cell numbers (Fig. 1G-I), indicating that So is required to maintain the Odd-positive prohemocyte population. Similar to our findings with Eya, So knockdown also increased lamellocyte numbers (Fig. 1J-L). In contrast, So knockdown in prohemocytes reduced crystal cell numbers (Fig. S3).

SIX overexpression phenocopies GATA1 overexpression in human erythroleukemia cells

To determine whether crosstalk between PSEDN members and the GATA network is conserved across taxa, we tested whether human SIX proteins could influence GATA1:FOG1 function in TF1 erythroleukemia cells, which can recapitulate early GATA1-dependent erythroid differentiation upon stimulation with erythropoietin (EPO) (Chrétien et al., 1996; Kitamura et al., 1989). Overexpression of GATA1 in erythroid progenitors produces a slow growth phenotype and promotes erythroid differentiation (Briegel et al., 1996). We similarly observed that lentivirus-mediated GATA1 expression reduced TF1 cell growth and triggered erythroid differentiation in standard TF1 growth media in the absence of exogenous EPO (Fig. 2A-D). Of the six human SIX genes, SIX1 and SIX2 are most closely related to each other and to *Drosophila* So (Kumar, 2009). TF1 cells transduced with either GATA1-, SIX1- or SIX2-expressing GFP-labeled lentivirus were monitored in long-term growth competition assays. As observed for GATA1, TF1 cells expressing SIX1 or SIX2 were rapidly outcompeted in mixed cell populations, consistent with reduced growth (Fig. 2A). In contrast, control GFP-expressing cells were maintained at a constant frequency during prolonged culture.

We next tested whether the reduced growth due to SIX1 or SIX2 overexpression was associated with erythroid differentiation in the absence of EPO, as we observed in cells overexpressing GATA1. Six days post-transduction, GATA1-, SIX1- or SIX2-expressing cell populations each exhibited an increased percentage of the erythroid cells, as assessed by transferrin receptor (CD71) and glycophorin A (CD235a) cell surface expression ($CD71^{hi}CD235a^{hi}$) (Gautier et al., 2016; Loken et al., 1987; van Lochem et al., 2004) (Fig. 2B,C), a decreased percentage of cells expressing the hematopoietic

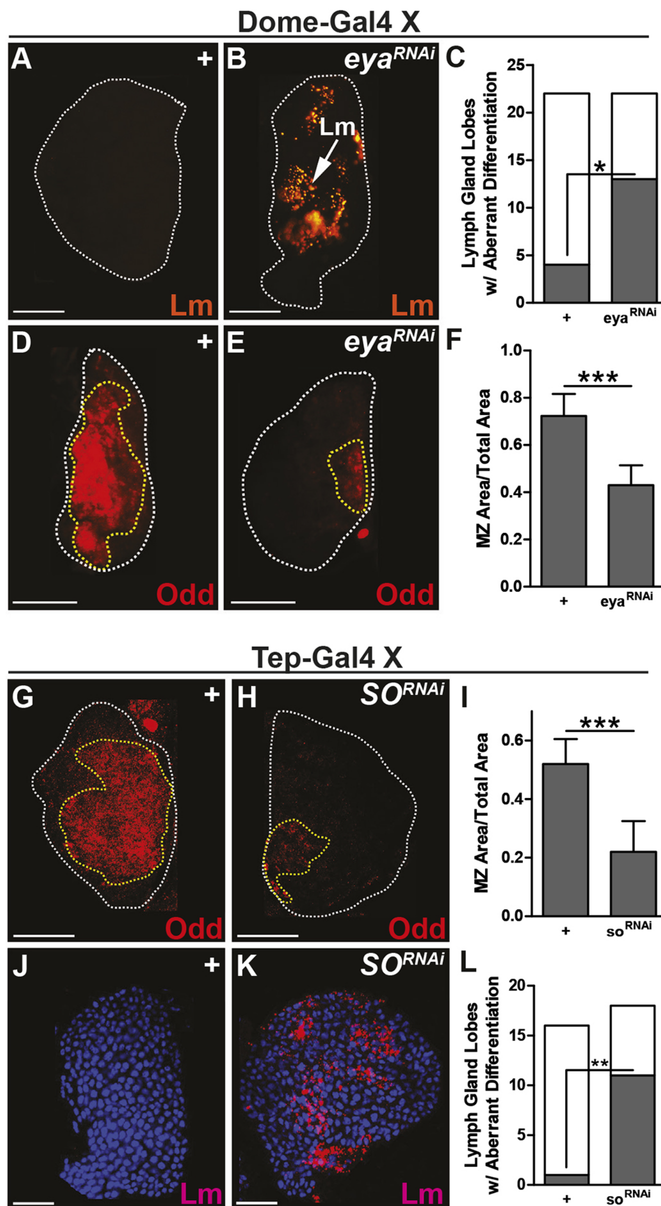

Fig. 1. Knockdown of Eya or So in the lymph gland prohemocytes increased lamellocyte numbers and decreased Odd-positive prohemocytes. (A-F) The MZ tissue-restricted *Dome-Gal4* driver was used to express *eya*^{RNAi} in prohemocytes. Analysis of late third larval instar larvae derived from *Dome-Gal4* driver flies crossed to wild type (+) versus *eya*^{RNAi}. (A,B) Lamellocytes (Lms) were identified using the specific marker L1 (Attila). (C) Histogram showing number of lymph gland lobes with aberrant lamellocyte differentiation was significantly increased in Eya knockdowns compared with controls. Gray versus white region shows the number of lymph gland lobes with aberrant lamellocyte differentiation versus wild-type phenotype, respectively. Fisher's exact test, *P<0.012; *Dome-Gal4* control (n=22); *Dome-Gal4* driving *UAS-eya*^{RNAi} (n=22). (D-F) The Odd-expression domain was significantly reduced in Eya knockdowns compared with controls. White dotted lines delineate the entire lymph gland; yellow dotted lines delineate the Odd-positive prohemocyte pool. (F) Histogram showing quantitation of Odd-positive domains. Data are mean±s.d., n=11, Student's t-test; ***P<0.0001. (G-L) The tissue-restricted *Tep-Gal4* driver was used to express *so*^{RNAi} in prohemocytes. Analysis of late third instar larvae derived from *Tep-Gal4* driver flies crossed to wild type versus *so*^{RNAi}. (G-I) Knockdown of So in lymph gland prohemocytes resulted in a significant decrease in the Odd-expression domain. (I) Histogram showing quantitation of Odd-positive domains (n=15) with standard deviation shown. Student's t-test; ***P=0.001. (J-L) Lamellocyte numbers were significantly increased in So knockdown compared with controls. Lamellocytes were identified by L1 marker and lymph glands were counterstained with DAPI (blue). (L) Histogram showing the numbers of lymph gland lobes with increased lamellocyte numbers (gray) versus wild-type phenotype (white). Fisher's exact test, **P<0.001; *Tep-Gal4* control (n=16); *Tep-Gal4* driving *UAS-so*^{RNAi} (n=18). Scale bars: 50 µm. n=number of lymph gland lobes.

stem-progenitor marker CD34 on the cell surface (Fig. 2B,C), increased mRNA levels of key erythroid genes, including HBB, ALAS2, SLC4A1, ANK1, KLF1 and SPTA1 (Fig. 2D), and elevated beta-hemoglobin protein levels (Fig. 2E). Thus, like GATA1, enforced expression of SIX1 or SIX2 produced phenotypes indicative of erythroid differentiation. Conversely, CRISPR-engineered *SIX1* knockout TF1 cells exhibited impaired erythroid differentiation in response to EPO stimulation, compared with controls (Fig. 2F-H), suggesting that SIX1 contributes to EPO-driven TF1 cell differentiation. No compensatory changes in the RNA levels of remaining SIX family members (SIX2-6) were observed in *SIX1* knockout cell lines (T.M.C. and T.J.K., unpublished observations).

We further tested whether EYA1, a human homolog of *Drosophila* Eya, could stimulate TF1 erythropoiesis. EYA1 overexpression resulted in increased numbers of CD71^{hi}CD235a^{hi} cells, reduced numbers of CD34^{+} cells (Fig. 3A-B), and increased hemoglobin protein levels (Fig. 3C, lane 1 versus 3) 10 days post-transduction in culture without EPO. As EYA proteins function as SIX co-regulators, we further tested whether SIX1 was necessary for EYA1 to stimulate TF1 erythropoiesis. Inducible CRISPRi-mediated knockdown of SIX1, which yielded >80% reduction in SIX1 protein levels, reduced EYA1-mediated induction of hemoglobin (Fig. 3C, lane 3 versus 4). Consistent with previous observations that EYA stabilizes SIX1 protein levels in the MCF7 breast cancer cell line (Patrick et al., 2009), we routinely observed slightly elevated levels of SIX1 in cells overexpressing EYA1 (Fig. 3C, lane 1 versus 3). Collectively, these data suggest EYA1 stimulates erythropoiesis and that EYA1 function is mediated in part via endogenous SIX1 protein.

SIX proteins modulate erythropoiesis in primary human hematopoietic stem-progenitor cells

We next tested whether SIX1 influences erythropoiesis in primary human hematopoietic stem-progenitor cells (HSPCs). HSPCs transduced with control versus SIX1-expressing lentiviruses were analyzed by flow cytometry after 3 days of culture in media supplemented with stem cell factor (SCF) and EPO. Overexpression of SIX1 in primary HSPCs resulted in an increased percentage of cells expressing erythroid surface markers (CD71^{hi} CD235a^{hi}) (Fig. 4A,B). Similar results were also observed for SIX2 overexpression (Fig. S4). In contrast, HSPCs transduced with SIX1 knockout CRISPR/Cas9 lentiviruses exhibited reduced erythroid cell generation compared with control transduced HSPCs (Fig. 4C,D). Thus, SIX1 enhances erythropoiesis in primary HSPCs.

SIX-mediated TF1 erythroid differentiation requires GATA1

As SIX1 OE phenocopied GATA1, we next tested whether stimulation of erythropoiesis by SIX1 was dependent on GATA1. Overexpression of SIX1 in wild-type versus *GATA1* knockout cells revealed that GATA1 was required for SIX1-driven generation of CD71^{hi}CD235a^{hi} erythroid cells, reduction of the CD34^{+} cell population, increased hemoglobin protein expression, and increased *KLF1*, *SPTA1*, *ANK1*, *HBB*, *ALAS2* and *SLC4A1* mRNA expression (Fig. 5A-D). These findings are consistent

Development (2020) 147, dev177022. doi:10.1242/dev.177022

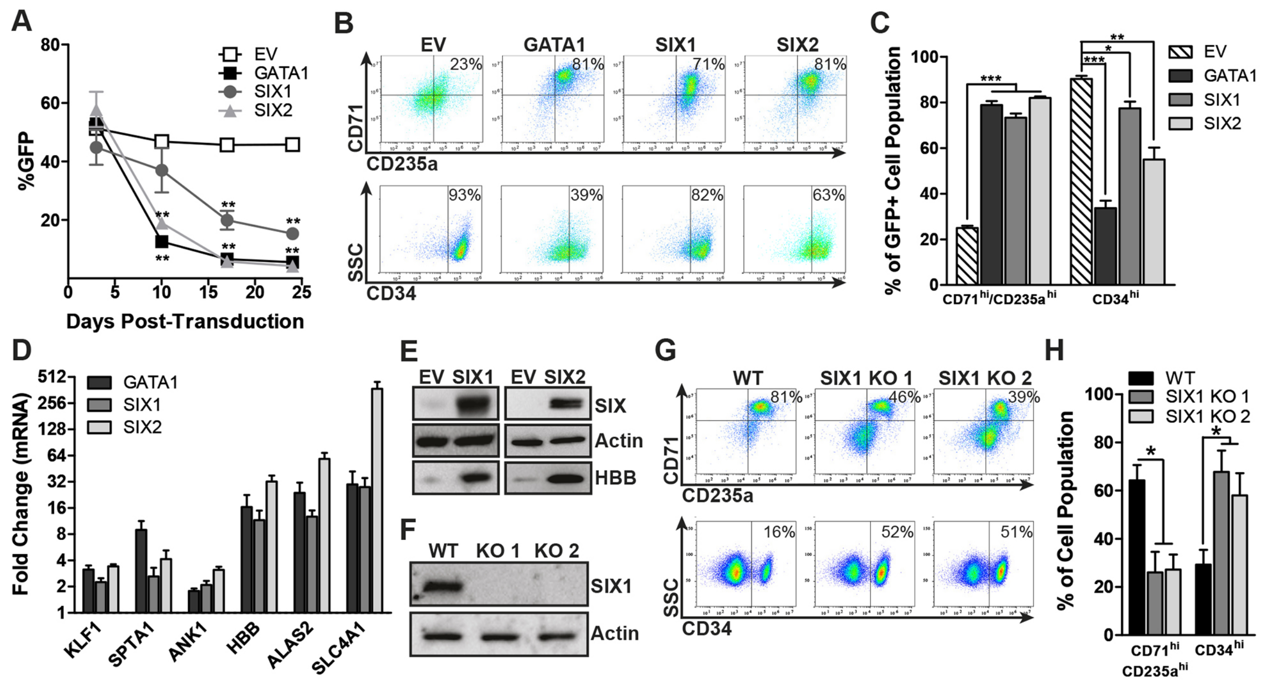

Fig. 2. SIX overexpression phenocopies GATA1 and drives TF1 cell erythroid differentiation. (A) GFP competition assays were conducted on TF1 cell populations transduced to MOI=0.5 (~50% GFP$^+$) with lentivirus expressing GFP alone (empty vector; EV), or lentivirus co-expressing GFP and GATA1, SIX1 or SIX2 (data are mean±s.e.m., *n*=3, ***P*<0.01 compared with EV). (B-D) TF1 cells were transduced with EV, GATA1 and SIX1, versus SIX2 lentivirus at MOI=2 (~90% GFP$^+$), and cultured in standard growth media. Cells were analyzed 6 days post-transduction. (B) Representative flow cytometric analysis for erythroid (CD71^{hi}CD235a^{hi}) and HSPC (CD34$^+$) markers. SSC, side scatter. (C) Quantitation of flow analysis performed using Flowlogic software. Data are mean±s.e.m. of the percentage of GFP$^+$ cells expressing the indicated surface markers, *n*=3, **P*<0.05, ***P*<0.01, ****P*<0.005. (D) Total RNA was harvested from cells, and levels of indicated erythroid gene RNA quantified by qRT-PCR and normalized to actin. Normalized RNA levels in EV-transduced cells were set to 1 for each gene. Increased RNA levels observed in GATA1-, SIX1- and SIX2-transduced cells was statistically significant compared with EV-transduced cells (*P*<0.05) for each gene. Data are mean±s.e.m., *n*=3. (E) Representative western blot images from SIX1- or SIX2-transduced TF1 cells after 6 days of culture in standard growth media. Total cellular lysates were harvested and analyzed using western blot for hemoglobin β (HBB), actin and either SIX1 or SIX2. (F) Representative western blot of protein extracts generated from wild type versus two independent SIX1 knockout (KO) TF1 cell lines probed for SIX1 or actin. (G) Representative flow cytometric analysis for erythroid cells (CD71^{hi}CD235a^{hi}) and CD34 in wild-type versus SIX1 knockout TF1 cell lines 3 days after EPO stimulation. (H) Quantitation of flow analysis performed using Flowlogic software. Data are mean±s.e.m., *n*=3, **P*<0.05.

with the SIX1-driven erythroid phenotype being mediated by GATA1. In contrast, the ability of SIX1 to increase CD235a surface expression appeared to be largely independent of GATA1 (Fig. 5A), suggesting SIX1 may regulate CD235a surface expression via multiple mechanisms.

SIX proteins control GATA levels across species

As SIX overexpression required GATA1 to stimulate erythropoiesis, we tested whether SIX overexpression altered GATA1 or GATA2 levels. Overexpression of SIX1 or SIX2 resulted in an ~50% increase in GATA1 and ≥50% decrease in GATA2 mRNA levels

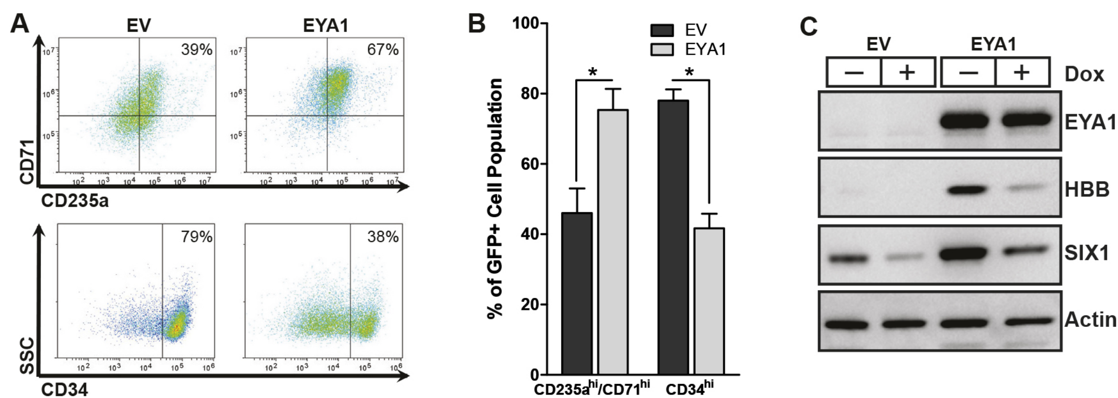

Fig. 3. EYA stimulates erythroid differentiation via SIX1. Wild-type TF1 cells were transduced to an MOI=2 (~90% GFP$^+$) with EV or EYA1-expressing lentiviruses cultured for 10 days in standard growth media. (A) Representative flow cytometry plots of transduced TF1s stained for erythroid markers (CD71^{hi}/CD235a^{hi}) and CD34. SSC, side scatter. (B) Quantitation of flow analysis performed in Flowlogic software. Data are mean±s.e.m. of the percentage of GFP$^+$ cells expressing indicated surface markers, *n*=3, **P*<0.05. (C) TF1 cells harboring doxycycline (DOX)-inducible *TRE*-dCas9-KRAB cells and SIX1-targeting sgRNA were cultured in the presence or absence of DOX for 4 days prior to transduction with EV versus EYA1-expressing lentiviruses at MOI=2 (~90% GFP$^+$). Cells were cultured for an additional 10 days in standard growth media supplemented with DOX every other day and then total protein lysates were harvested and analyzed via western blot for EYA1, SIX1, HBB and actin.

Development (2020) 147, dev177022. doi:10.1242/dev.177022

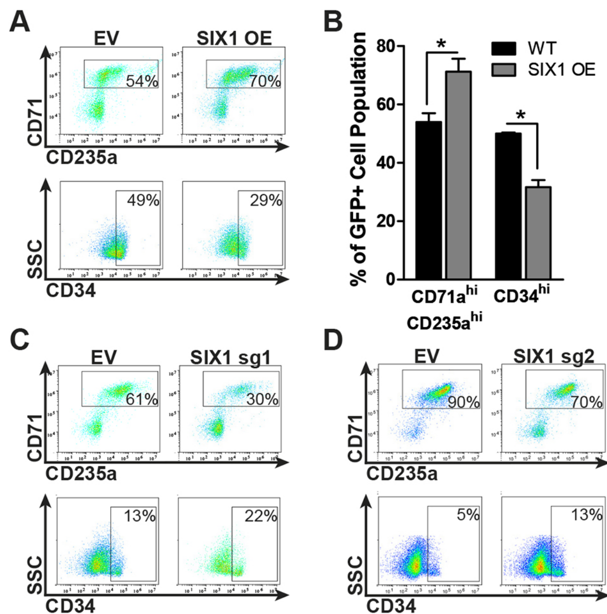

Fig. 4. SIX1 overexpression drives erythroid differentiation, whereas CRISPR/Cas9-mediated SIX1 knockout inhibits erythroid differentiation in primary human CD34⁺ HSPCs. (A) Representative flow cytometric analysis for erythroid ($CD71^{hi}CD235a^{hi}$) cells following SIX1 overexpression. Three days after transduction with lentiviruses, EV-transduced versus SIX1 OE-transduced HSPCs were cultured in the presence of SCF and EPO for 3 days prior to analysis for cell surface expression of erythroid surface markers CD71 and CD235a or the HSPC marker CD34. SSC, side scatter. (B) Quantitation of three independent experiments. Data are mean percentage of GFP^+ cells expressing indicated surface markers±s.e.m., **P*<0.05. (C,D) Flow cytometric analysis for erythroid ($CD71^{hi}CD235a^{hi}$) cells following SIX1 knockout. Three days post-transduction with lentivruses co-expressing Cas9 and a SIX1 targeting sgRNA, HSPCs were transferred to cultures containing SCF and EPO for 6 days prior to analysis. Erythroid marker and CD34 surface expression was assessed in EV-transduced HSPCs versus two distinct SIX1 targeting sgRNAs, (C) sgRNA1 and (D) sgRNA2.

6 days post-transduction (Fig. 6A). Likewise, SIX1 or SIX2 overexpression reduced GATA2 protein levels by >80% and elevated GATA1 protein levels ~50% and 70%, respectively (Fig. 6B,C). The observed changes in GATA expression levels were similar to those observed in TF1 cells following EPO stimulation (Fig. S5). Thus, in the absence of exogenous EPO, enforced expression of SIX1 and SIX2 triggers changes in GATA1 and GATA2 expression consistent with the GATA switch.

To determine whether SIX regulates GATA levels across species, we tested whether manipulation of So levels altered Srp/GATA levels *in vivo* in *Drosophila* larval lymph glands. Overexpression of So/SIX (*so^GOF*) in prohemocytes using the *Tep-Gal4* increased Srp/GATA protein levels. Conversely, So/SIX RNAi knockdown of So (*so^RNAi*) reduced Srp/GATA protein levels (Fig. 6D-G). Thus, Srp/GATA levels in *Drosophila* larval lymph glands are dependent upon So/SIX. Taken together, these observations demonstrate that SIX regulates GATA levels across taxa. Previously, we demonstrated that Srp overexpression in prohemocytes reduces prohemocytes and increased lamellocytes (Gao et al., 2013). Consistent with increased levels of Srp/GATA observed in So overexpression, we observed a significant reduction of Odd-positive cells (Fig. 6H-J) and increased numbers of lamellocytes (Fig. 6K-M) in *so^GOF* lymph glands. Thus, gain of So function increases lamellocytes and reduces prohemocytes likely by increasing the level of Srp expression.

Human SIX proteins interact with GATA1 and stimulate GATA1 activity

Using SIX biotin proximity labeling (Roux et al., 2012) in TF1 cells, we identified GATA1 as a member of the SIX proximal interactome. As shown in Fig. 7A, GATA1 was selectively enriched in streptavidin pulldowns from BirA*-SIX2 compared with BirA* control transduced cells, indicating that GATA1 is proximal to SIX2. We therefore tested whether SIX proteins co-immunoprecipitate GATA1. GATA1 was co-expressed in HEK293T cells with MYC-tagged SIX1 or MYC-tagged SIX2. Anti-MYC selectively co-immunoprecipitated GATA1 from extracts containing MYC-SIX1 or MYC-SIX2 (Fig. 7B,C), demonstrating that SIX1 and SIX2 can physically interact with GATA1 complexes. To examine the ability of endogenous SIX proteins to interact with GATA1, we performed proximity ligation assay (PLA) (Weibrecht et al., 2010) in TF1 cells. As shown in Fig. 7D, the PLA assay revealed an association between endogenous SIX1 and GATA1 in wild-type TF1 cells, that was lost in SIX1 knockout cells. Taken together, our results demonstrate that human SIX proteins associate with GATA1 complexes.

To determine the consequences of SIX-GATA1 interaction on GATA1 transcriptional output, HEK293T cells were co-transfected with a minimal GATA-dependent reporter gene containing three copies of the GATA-binding site (A/TGATAA/G) (Collavin et al., 2004) in the presence or absence of GATA1 and SIX1 or SIX2 expression constructs. Enforced expression of GATA1 alone resulted in a ninefold stimulation of reporter gene activity, whereas enforced expression of either SIX1 or SIX2 alone resulted in a two- to threefold stimulation of reporter activity. Co-expression of GATA1 with either SIX1 or SIX2, however, resulted in 30- to 40-fold stimulation of activity (Fig. 7E). Collectively, these findings suggest that SIX1 and SIX2 can bind to GATA1 protein complexes and increase GATA1-mediated transcription (Fig. 7E).

DISCUSSION

Here, we report novel functional interactions between the GATA network and the PSEDN, two major developmental regulatory hubs conserved from fly to human (Davis and Rebay, 2017; Fossett, 2013; Katsumura et al., 2017; Tadjuidje and Hegde, 2013). Using an SSNC screen based on the requirement for Ush-Srp/FOG-GATA to block lamellocyte differentiation, we discovered a genetic interaction between a key GATA network member, FOG, and the PSEDN in *Drosophila*. We subsequently showed that both Eya/EYA and So/SIX PSEDN members are required in prohemocytes to block lamellocyte differentiation *in vivo* and to maintain the prohemocyte pool. Our findings suggest that So/SIX and Eya/EYA act in concert with Srp-Ush/GATA-FOG to limit lamellocyte differentiation. In contrast, knockdown of So/SIX reduced crystal cell numbers, suggesting PSEDN members may exert distinct functions in a cell context-dependent manner. Generation of crystal cells is dependent upon Srp/GATA-Lz (Lonzenge)/RUNX and opposed by Ush (Fossett et al., 2003; Muratoglu et al., 2006, 2007; Waltzer et al., 2002). Thus, although So is required for Srp/Ush to maintain prohemocytes and block lamellocyte differentiation, it may function with Srp/Lz in crystal cell differentiation. Although the role of the PSEDN is well studied in retinal determination in the fly, its role in hematopoiesis has not been previously reported.

Using erythropoiesis, a robust model for mammalian GATA function where GATA1-FOG1 drives central features of erythroid differentiation, we demonstrate that human PSEDN members SIX and EYA enhance GATA1-dependent cell phenotypes and transcriptional output *in vitro*. Overexpression of SIX1 or SIX2 in

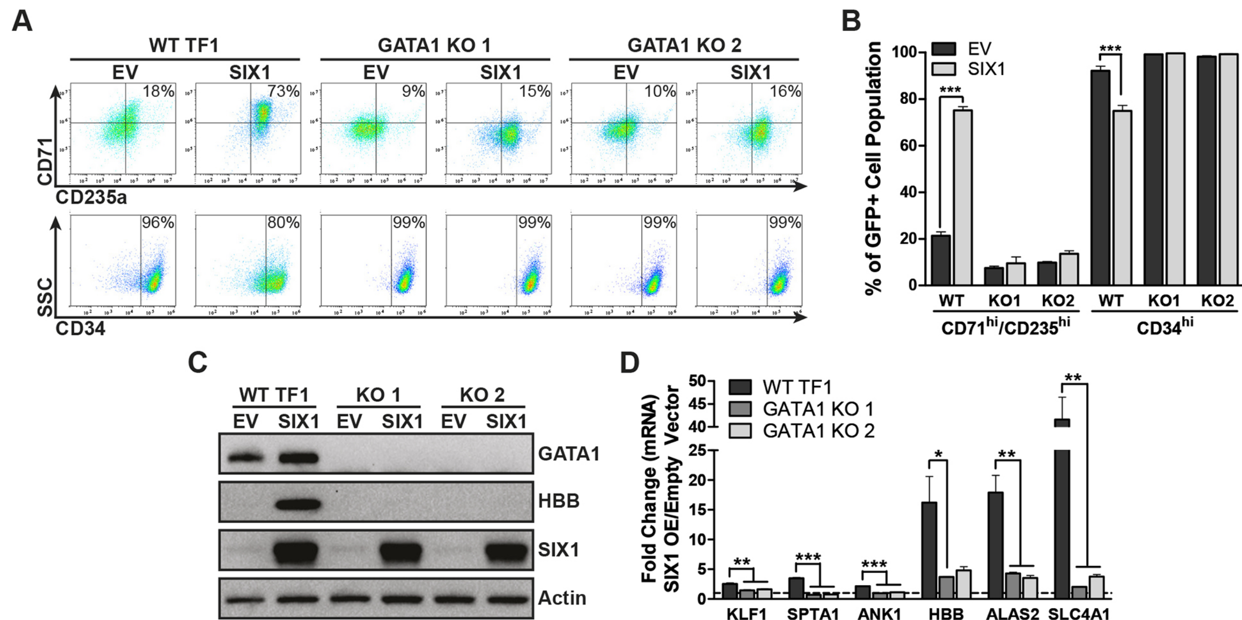

Fig. 5. SIX1 stimulates erythroid differentiation via GATA1. TF1 cells cultured in standard growth media were transduced at MOI=2 (~90% GFP$^+$) with empty vector control (EV) versus SIX1-expressing lentiviruses and analyzed 6 days post-transduction. (A) Representative flow cytometry plots of wild-type versus two independent GATA1 knockout (KO) TF1 cell lines stained for erythroid markers (CD71^{hi}/CD235a^{hi}) and CD34. (B) Quantitation of flow analysis performed in Flowlogic software. Data are mean percentage of GFP$^+$ cells expressing indicated surface markers±s.e.m., *n*=3, ****P*<0.005. (C) Representative western blot of protein extracts generated from wild-type versus GATA1 KO TF1 cells probed with anti-HBB, anti-GATA1, anti-SIX1 and anti-Actin antibodies. (D) qRT-PCR analysis of erythroid gene panel. Total RNA was harvested from cells and levels of indicated erythroid gene RNA quantified by qRT-PCR and normalized to actin. For each cell line, relative RNA level observed in EV transduced cells was set to 1 (dotted line). Data are mean fold change in SIX1-overexpressing cells relative to corresponding EV±s.e.m., *n*=3, **P*<0.05, ***P*<0.01, ****P*<0.005.

TF1 erythroleukemia cells phenocopied GATA1 overexpression, triggering erythropoiesis in the absence of exogenous EPO. In primary human HSPCs, enforced SIX expression enhanced EPO-stimulated erythropoiesis. In contrast, SIX1 knockout impaired erythropoiesis, suggesting endogenous SIX1 contributes to human erythropoiesis *in vitro*. Our observation that SIX overexpression could not stimulate erythropoiesis in the absence of GATA1 indicates that SIX proteins function in conjunction with or upstream of GATA1. This conclusion is supported by our finding that SIX overexpression increases GATA1 and decreases GATA2 RNA and protein levels, as occurs during EPO-stimulated erythropoiesis (Kaneko et al., 2010). Intriguingly, the ability of PSEDN members to regulate GATA levels is conserved across taxa. Knockdown and overexpression of So/SIX levels produced corresponding changes in Srp expression levels, revealing Srp/GATA levels are dependent upon So/SIX in fly prohemocytes. Increased Srp/GATA levels following So/SIX overexpression likely underlies our observation that So/SIX gain of function increased lamellocyte differentiation and reduced the prohemocyte population, as Srp/GATA overexpression has previously been shown to increase lamellocyte differentiation at the expense of prohemocytes (Gao et al., 2013). If the physical interaction we observed between human SIX and GATA is conserved in *Drosophila*, So/SIX binding to Srp/GATA to increase its function may also contribute to the mechanism that drives increased lamellocyte differentiation in So gain-of-function fly lymph glands.

In addition to demonstrating that the PSEDN and GATA networks exhibit genetic and functional interactions, we show that human SIX proteins can associate with GATA1 complexes and stimulate GATA1-mediated transcription of a minimal GATA-dependent reporter gene. Our observations may reflect direct binding between SIX and GATA1 or indirect protein-protein binding orchestrated via a multiprotein complex. As SIX1 and SIX2 have unique C-terminal domains, our results implicate the conserved domains (N terminus SIX domain and homeobox domain) in mediating the physical and functional interactions with GATA1, thereby raising the possibility that additional SIX family members might also interact with GATA1. Humans possess six GATA proteins and six SIX proteins, which regulate specification, survival, differentiation and function of multiple tissues. The extent to which additional GATA and SIX family members (as well as other GATA-FOG network and PSEDN members) interact to coordinately regulate gene expression may provide novel insights into fundamental developmental processes. Identifying the repertoire of protein interactions between these two families will reveal key mechanisms that drive normal lineage-specific development and malignancies, as specific SIX-GATA interactions may fine-tune GATA output.

A role for vertebrate PSEDN members in erythropoiesis has not previously been reported. Here, we observed that EPO failed to fully stimulate erythroid phenotypes in human TF1 cells and HSPCs lacking SIX1. Furthermore, the absence of SIX1 reduced the ability of EYA1 overexpression to stimulate features of erythroid differentiation, indicating that EYA1 stimulates TF1 cell erythropoiesis in part via SIX1. In addition to SIX1, human hematopoietic cells express additional SIX family members (data not shown); this redundancy may partially compensate for loss of SIX1 function in TF1 cells and may prevent overt hematopoietic phenotypes in knockout mice lacking a single SIX gene (Laclef et al., 2003; Li et al., 2003; Zhang et al., 2018). Additionally, SIX proteins may primarily function *in vivo* in hematopoiesis as accessory factors to enhance GATA1 function. Thus, the requirement for SIX may not be detected unless the system is stressed. Recent work demonstrating an essential role for SIX1 in MLL-AF9 leukemogenesis, also included data suggesting

Development (2020) 147, dev177022. doi:10.1242/dev.177022

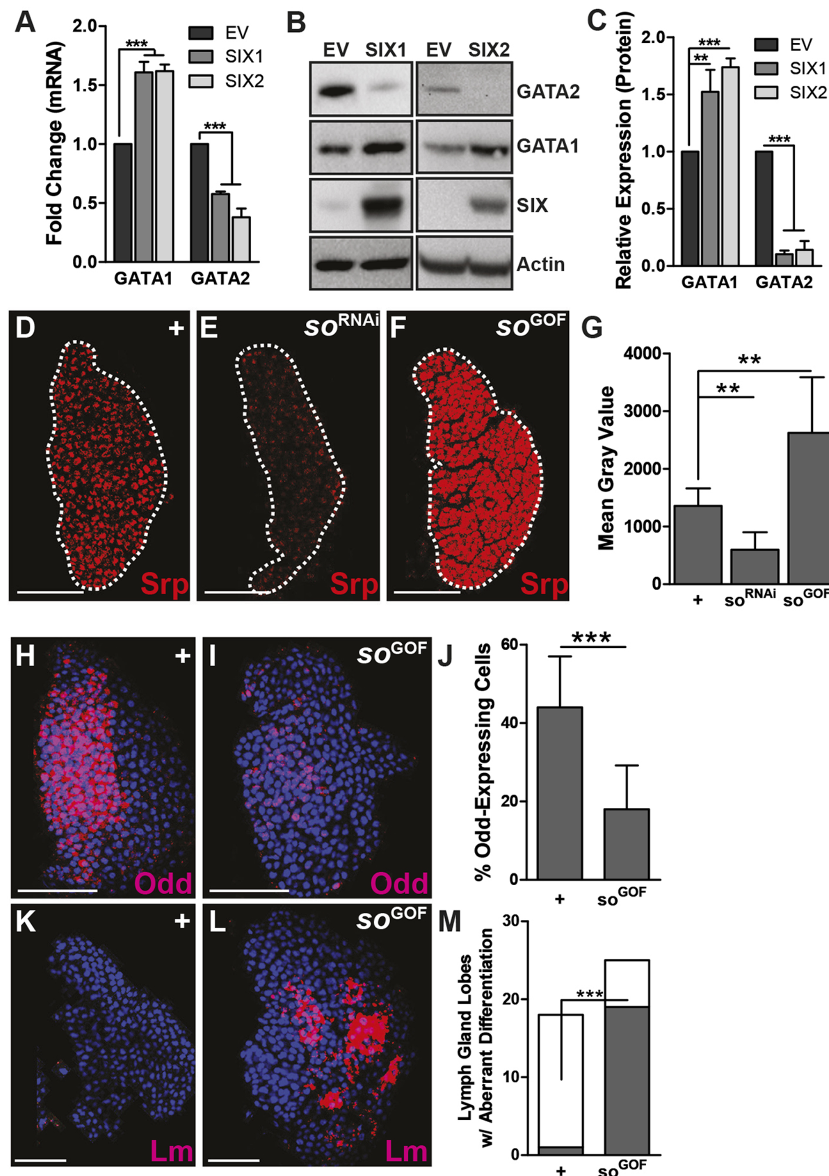

Fig. 6. SIX/So proteins regulate GATA/Srp expression levels. (A) Analysis of GATA1 and GATA2 RNA levels following SIX overexpression. Total RNA was harvested from SIX1 and from SIX2 versus EV-transduced TF1 cells, and analyzed by qRT-PCR. GATA levels were normalized to actin and fold change calculated relative to EV control samples. Data are mean±s.e.m., *n*=3, all values were significantly different compared with EV. ****P*<0.005. (B) Representative blots from SIX1- or SIX2-transduced TF1 cells after 6 days in standard growth media culture post-transduction. Protein lysates were harvested and analyzed by western blot for GATA2, GATA1, SIX1, SIX2 and Actin. (C) Data are mean fold change of densitometric mean above EV after normalization to Actin±s.e.m. (***P*<0.01, ****P*<0.005). (D-F) The tissue-restricted *Tep-Gal4* driver was used to knockdown So (so^{RNAi}) or overexpress *so* (so^{GOF}) in prohemocytes. Lymph glands are outlined with a dotted line. Srp levels were monitored using a Srp-specific antibody; (D) control (+), (E) so^{RNAi}, (F) so^{GOF}. (G) Histogram showing that so^{RNAi} significantly reduced Srp levels by >50% (*n*=10; ANOVA, ***P*<0.01), whereas so^{GOF} significantly increased Srp levels approximately twofold (*n*=10; ANOVA, ***P*<0.01). Data are mean gray value/lobe for each genotype±s.d. (see Materials and Methods). (H-M) The tissue-restricted *Tep-Gal4* driver was used to overexpress wild-type *so* (so^{GOF}) in prohemocytes. Lymph glands were dissected from late third instar larvae derived from *Tep-Gal4* driver flies crossed to wild type (+) or so^{GOF} and lymph glands were counterstained with DAPI (blue). (H-J) The percentage of Odd-positive cells was significantly reduced in so^{GOF} compared with controls. (J) Histogram showing quantitation of percentage of Odd-positive cells (*n*=10). Data are mean±s.d. Student's *t*-test; ****P*<0001. (K-M) Lamellocytes were identified using the specific marker L1. (M) Histogram showing lamellocytes significantly increased in so^{GOF} compared with controls. Gray region indicates number of lymph gland lobes with aberrant lamellocyte differentiation and white indicates number of lobes with the wild-type phenotype. *Tep-Gal4 control* (*n*=18); *Tep* driving *UAS-so*^{GOF} (*n*=25). Fisher's exact test; ****P*<0001. Scale bars: 50 µm. *n*=number of lymph gland lobes.

reduced numbers of megakaryocyte-erythrocyte progenitors in *Six1* knockout mice during their analysis of normal hematopoiesis, although the decrease did not reach statistical significance (Zhang et al., 2018).

Our finding that SIX proteins associate with and modulate GATA protein levels, suggests that multiple layers of interaction exist between these evolutionarily conserved developmental networks. The ability of SIX1 to affect GATA family member expression has previously been reported. SIX1 activates GATA2 and represses GATA3 in Hodgkin's lymphoma (Nagel et al., 2015) but stimulated GATA2 and repressed GATA3 in RAS-transformed fibroblasts (De Lope et al., 2019), demonstrating the importance of cellular context in shaping SIX1 modulation of individual GATA members and suggesting a potential commonality of SIX proteins in orchestrating GATA switches. Conversely, GATA1 has been shown to regulate SIX2 expression in mouse embryonic kidney cells, and GATA-binding domains have been shown to contribute to Six1 expression via a conserved Six1 rostral PPR (pre-placodal region) enhancer (Fujimoto et al., 2013), suggesting bi-directional regulatory interactions (Xia et al., 2017).

In summary, we provide evidence that the PSEDN can genetically and physically interact with the GATA network across taxa to regulate GATA-dependent cell functions. PSEDN-GATA interactions revealed in this study may also have important implications in disease, as both GATA1 and SIX1 are emerging as important factors in human cancers (Blevins et al., 2015; Caldwell et al., 2013; Coletta et al., 2008; Micalizzi et al., 2009; Zhao et al., 2014; Zheng and Blobel, 2010). Overexpression of GATA1 or SIX1 drives EMT in breast cancer cells (Li et al., 2015; Micalizzi et al., 2009) and confers chemoresistance to multiple types of cancer cells (Behbakht et al., 2007; Caldwell et al., 2013; Li et al., 2013). Our observations provide novel mechanisms by which these networks may cooperate to regulate normal development and malignant processes in cancer biology.

MATERIALS AND METHODS

Fly strains and genetic analyses

The w^{1118} strain served as the wild-type stock for these studies (Gao et al., 2009). *Dome-Gal4*, *Tep4-Gal4* and *MSNF9mo-DsRed* (*MSN-C*) have been previously described (Baldeosingh et al., 2018) and we gratefully recognize

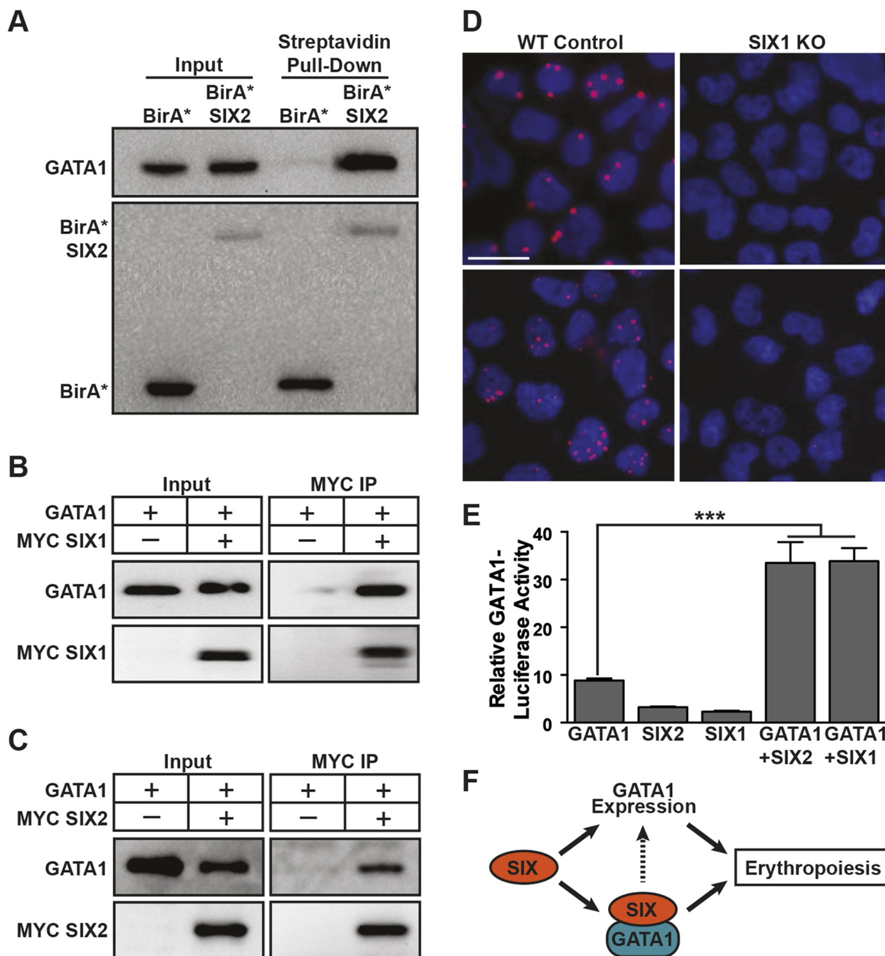

Fig. 7. SIX proteins interact with GATA1 and stimulate GATA1 transcriptional output. (A) Western blot analysis of total cell extracts (input) versus streptavidin pull-downs generated from biotin-labeled TF1 cells transduced with BirA* versus BirA*-SIX2 probed with anti-GATA1 or anti-BirA antibodies as indicated. (B,C) Western blot analysis of anti-MYC immunoprecipitation assays conducted on total cell lysates generated from HEK293T cells co-transfected with GATA1 and MYC-SIX1/MYC-SIX2 versus empty vector. Total cell extract (input) versus anti-MYC-IP samples probed with anti-GATA1 or anti-MYC antibodies. (D) PLA assays testing association of SIX1 and GATA1. No signal was observed in SIX1 KO compared with control cells with endogenous SIX1. Scale bar: 25 µm. Images from two independent experiments shown. (E) HEK293T cells were co-transfected with GATA1-luciferase reporter plasmid, SV40-Renilla luciferase and empty vector, SIX1, SIX2 or GATA1 expression plasmids. Firefly luciferase activity was normalized to *Renilla* luciferase activity. EV-transfected cultures were set to 1. Data plotted are representative set of quadruplicates with s.d. indicated (***$P<0.001$). (F) Schematic showing that SIX1 can promote GATA1-mediated erythropoiesis via two mechanisms: (1) increasing GATA1 expression; and (2) binding to GATA1 complexes to enhance GATA1 function. The latter physical interaction may contribute to increased GATA1 expression via GATA1 autoregulation (dashed arrow). Given conserved crosstalk between GATA and SIX across taxa, similar SIX-GATA interactions may orchestrate distinct developmental contexts.

generous gifts of fly stocks from colleagues in the Acknowledgements. The following strains were obtained from the Bloomington Stock Center: $eya^{cli\text{-}2II}cn^{1}$ bw^{1} sp^{1}/CyO, so^{1}, so^{2}, ey^{1}, ey^{2}, b^{1} dac^{1} pr^{1} cn^{1} wx^{wxt} bw^{1}/CyO, dac^{9} $pk^{sple\text{-}3}/CyO$, *Df(2R)cn-S3* $Dp(?;2)bw^{D}$, so^{3} bw^{D}/CyO, y^{1} sc^{*} v^{1}; $P\{y^{+t7.7}$ $v^{+t1.8}$*=TRiP.HMS01441attP2* (so^{RNAi}), y^{1} v^{1}; *P{TRiP.JF03160}attP2* (eya^{RNAi}), *P{ry[+t7.2]=sevRas1.V12}FK1*, st^{1} $trx^{1}/TM1$, brm^{2} trx^{E2} $ca^{1}/$ *TM6B*, Tb^{1} ca^{1}, *w**; dac^{E462} $P\{r^{[+t7.2}$*=neoFRT}40A/CyO*, *w**; $P\{w^{+mC}$*=UAS-so.P}1*., *yw;* ush^{vx22}/CyO y^{+} has been previously described (Gao et al., 2009). The *yw;* ush^{vx22}, *MSN-C/CyO* y^{+} was created using standard recombination procedures and the SSNC screen was previously described (Baldeosingh et al., 2018). Gene expression analyses were conducted using lymph glands from late third instar larvae (collected 120 to 144 h after egg laying) as previously described (Gao et al., 2009). *Dome-Gal4* is expressed in non-hematopoietic tissues during development and when used to drive certain master regulators can lead to early larval death by disrupting essential developmental programs (Baldeosingh et al., 2018).

Screen for factors that genetically interact with *ush*

The *yw; ushvx22*, *MSN-C/CyO y+* stock enabled us to rapidly screen for genes that genetically interact with *ush* to block lamellocyte differentiation. The *MSN-C* lamellocyte marker was used to identify larvae with an increase in circulating lamellocytes using fluorescence microscopy. Scoring was carried out on late third instar wandering larvae, which were cultured at 23°C. Larvae were placed on a slide with a drop of PBS and observed under fluorescent microscopy using a Zeiss Axioplan microscope. *MSN-C* is also constitutively active in larval muscle and serves as a marker for larvae that carry the *ushvx22*, *MSN-C* chromosome. The *yw; ushvx22*, *MSNC/ CyO y+* stock was crossed to each of the 100 large multi-gene deficiencies that map to Chromosome 2L, to produce *ush/Df(2L)* trans-heterozygotes. The deficiency that encompasses *ush*, which maps between 21D1 and 21E2 on chromosome 2L, however, could not be scored because *ush* homozygotes are embryonic lethal. Individual genes mapping to deficiencies scoring positive for increased lamellocyte production were then tested by crossing fly stocks carrying null alleles of individual genes to the *yw; ushvx22*, *MSNC/ CyO y+* stock.

Immunofluorescence

Dissection and fixation of larval lymph glands were performed as previously described (Gao et al., 2009). We gratefully recognize the generous gifts of specific *Drosophila* antibodies from colleagues in the Acknowledgements section. Antibody dilutions were as follows: rabbit anti-Odd (Ward and Skeath, 2000), 1:4000; mouse anti-Attila (L1) (Kurucz et al., 2007), 1:50; rabbit anti-Serpent, 1:8000 (Gao et al., 2013); rabbit anti-U-shaped, 1:4000 (Gao et al., 2009); and rabbit anti-prophenoloxidase A1 (anti-PPO), 1:100 (Müller et al., 1999). Alexa Fluor 555-conjugated secondary antibodies (Thermo Fisher Scientific, A32727 or A32732) were used at a 1:2000 dilution.

For all experiments, at least 18 lobes were examined (nine control and nine experimental samples). In all experiments, control and experimental lymph glands were processed side by side using the same batch of reagent, including formaldehyde and primary and secondary antibodies to guard against variation due to slight differences in reagent preparation. For lamellocytes, wild-type animals contain on average one lamellocyte per lobe. Lymph gland lobes were stained for the lamellocyte marker Attilla, and counterstained with DAPI to visualize the whole lobe. Lamellocytes were hand counted under the microscope at 40× magnification. Lobes with more than five lamellocytes were scored as having aberrant lamellocyte differentiation. Statistical significance was evaluated using aberrant differentiation as a categorical variable for experimental and control samples in 2×2 contingency tables and *P*-values were calculated using Fisher's Exact test (Gao et al., 2011, 2013, 2014). To determine relative protein expression levels, lymph glands were scanned using confocal microscopy with the Cy3 filter. The mean gray value

DEVELOPMENT

Development (2020) 147, dev177022. doi:10.1242/dev.177022

for each 3 μm optical slice was determined across the entire lymph gland lobe using ImageJ quantitation software. A series of approximately four to seven measurements representing the entire width of the lobe was averaged and reported as the average mean gray value. This was repeated for at least nine lymph gland lobes and compared across genotypes. Statistical significance was determined using ANOVA. The size of the Odd$^+$ domain was determined by measuring the area of Odd-expressing cells using the Axiovision or ImageJ outline spline interpolation feature. The lymph gland was photographed in bright field and the area of the total lymph gland lobe was measured again using the outline spline interpolation feature. The relative size of the Odd$^+$ domain was determined by dividing the Odd$^+$ expression domain area by the total area of the lymph gland lobe. Blood cell counts were determined using ImageJ quantitation software and the percentage was determined by dividing the number of cells expressing a particular protein by the number of DAPI-stained nuclei. The statistical significances of expression domain and percentage cell counts were evaluated using Student's *t*-test.

Plasmids

All plasmids and primers used in this study are listed in Tables S1 and S2. Gene sequences used for lentivector expression constructs are also provided in Table S3. Gene expression constructs were generated by PCR or G-blocks synthesis and cloned into the GFP-marked lentivector pWCC43 (Kim et al., 2015). BirA* was PCR amplified from pcDNA3.1 mycBioID plasmid (a gift from Kyle Roux, Sanford Children's Health Research Center, USA; Addgene plasmid #35700) (Roux et al., 2012). Expression plasmids created for this study were SIX1, pTJK299 (Addgene #138000); SIX2, pTJK422 (Addgene #138002); GATA1, pTJK482 (Addgene#138001); EYA1, pTJK460 (Addgene #138003); 3xMYC-SIX1, pTJK438 (Addgene#138004); 3xMYC-SIX2, pTJK477 (Addgene #138005); BirA*, pTJK368 (Addgene#138006); and BirA*-SIX2, pTJK421 (Addgene#138007). For gene knockout, GATA1 and SIX1 sgRNAs were synthesized as primers (Integrated DNA Technologies) and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (a gift from Feng Zhang, MIT, Cambridge, MA, USA; Addgene plasmid #62988) (Ran et al., 2013) to create pTJK637 (Addgene #138010) and pTJK635 (Addgene #138009), respectively, or plentiCRISPRv2GFP (a gift from David Feldser, University of Pennsylvania, Philadelphia, PA, USA; Addgene #82416) (Walter et al., 2017), to create pTJK643 (Addgene #138011) and pTJK644 (Addgene #138012). For CRISPRi, SIX1 sgRNA was cloned into the plenti SpBsmBI sgRNA Puro vector (a gift from Rene Maehr, University of Massachusetts Medical School, Worcester, MA, USA; Addgene plasmid #62207) (Pham et al., 2016) to create pTJK642 (Addgene #138013) or into a modified version of plenti SpBsmBI sgRNA Puro vector (pTJK459, Addgene #138008) created by site-directed mutagenesis (QuikChange kit, Agilent) to insert U-A flip and extended hairpin (cgttt*A*agagcta*TGCTG*gaaa*CAGCA*tagcaagtt*T*aaata) (Chen et al., 2013) to create pTJK475 (Addgene #138014). pHAGE TRE dCas9-KRAB was a gift from Rene Maehr and Scot Wolfe (University of Massachusetts Medical School, Worcester, MA, USA) (Addgene plasmid #50917) (Kearns et al., 2014).

Cell culture and transduction

TF1 human erythroleukemia cells (CRL-2003; American Type Culture Collection) were cultured in standard growth media (RPMI, 10% FBS supplemented with GM-CSF) as previously described (Kim et al., 2015), except recombinant human GM-CSF was from TONBO Bioscience. HEK293T cells (American Type Culture Collection) were cultured in DMEM media (CellGro) containing 10% FBS. TF1 lentivirus transductions were conducted in the presence of 8 ng/ml polybrene. Triplicates represented in histograms correspond to experiments performed using cells from three independent lentivirus transductions.

Lentivirus transduction and erythroid differentiation of human primary CD34$^+$ HSPCs

Human CD34$^+$ HSPCs from normal adult donors were obtained from the Cellular Therapy and Cell Processing Facilities (Fred Hutchinson Cancer Center, Seattle, WA, USA). To overexpress or knockout SIX1 in primary human CD34$^+$ HSPCs, lentivirus transductions were conducted with 1 mg/ml Kolliphor P407 (Sigma-Aldrich) and 10 μM 16,16-dimethyl-prostaglandin E2 (dmPGE2; Abcam) in StemSpanSFEM medium (Stemcell Technologies) containing 100 ng/ml human stem cell factor (SCF; PeproTech), 20 ng/ml thrombopoietin (TPO; PeproTech) and 100 ng/ml FMS-like tyrosine kinase receptor 3 ligand (FLT3L; PeproTech) (Kim et al., 2015; Masiuk et al., 2019). CD34$^+$ HSPCs were transduced with lentiviruses at MOI=100, which is calculated based on the transduction units in HEK293T cells. Transduced HSPCs were cultured in StemSpanSFEM medium (Stemcell Technologies) containing 100 ng/ml human stem cell factor (SCF; PeproTech) and 1 U/ml erythropoietin (EPO; TONBO Bioscience) to induce erythropoiesis. After 6 days culture in EPO media, erythroid differentiation of the GFP$^+$ cell population was quantified by flow cytometry analysis for cell-surface marker expression. SIX1 knockout in CD34 cells was conducted by cloning SIX1 targeting guide RNA sequences into plentiCRISPRv2GFP, a gift from David Feldser (University of Pennsylvania, Philadelphia, PA, USA; Addgene #82416) (Walter et al., 2017).

Flow cytometry

TF1 cells were stained using monoclonal antibodies against CD235a (551336, BD Biosciences), CD71 (25-0719-42, eBioscience) and CD34 (555824, BD Biosciences) assessed by flow cytometry (Accuri C6, BD Biosciences) and data analyzed using FlowLogic Software (Milltenyi Biotec) as previously described (Kim et al., 2015). A representative gating strategy is shown in Fig. S6. For GFP competition assays, lentivirus-transduced (MOI=0.5) TF1 cells containing ∼50% GFP$^+$ cells on day 3 post-transduction, were assayed weekly by flow cytometry to determine the percentage of GFP$^+$ cells.

Protein isolation and western blot analysis

Protein lysates from transduced TF1 cells were quantitated and analyzed by western blot as previously described (Kim et al., 2015) using anti-SIX1 (12891, Cell Signaling Technology; 1:2000), -SIX2 (ab111827 Abcam; 1:1000), -HBB (sc-21757, Santa Cruz Biotechnology; 1:200), -GATA1 (sc-266, Santa Cruz Biotechnology; 1:200), -GATA2 (sc-267, Santa Cruz Biotechnology; 1:200), -EYA1 (H00002138-A01, Abnova; 1:500), -MYC epitope tag (2276S, Cell Signaling Technology; 1:2000), -BirA (NBP2-59939, Novus Biologicals; 1:500) and -β-Actin (8457S, Cell Signaling Technology; 1:5000), with HRP-conjugated secondary antibodies from Jackson ImmunoResearch. Signal was detected using enhanced chemiluminescence (ECL; Thermo Fisher Scientific) and quantitated using ImageJ (Schindelin et al., 2012; Schneider et al., 2012).

RNA analysis

Total RNA was extracted and analyzed as previously described (Kim et al., 2015). Reverse transcription was conducted with 1.0-1.5 µg RNA using a High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific). qPCR was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) on an Applied Biosystems Quant-Studio 6 Flex according to manufacturers' protocol and analyzed using QuantStudio Real-Time PCR Software (Thermo Fisher Scientific).

Luciferase assays

HEK293T cells cultured in 24-well dishes were transfected using Lipofectamine 2000 (Thermo Fisher Scientific). All wells were transfected with equal amounts of total DNA, comprising pGL3 GATA Luc (a gift from Licio Collavin and Giannino Del Sal, University of Trieste, Trieste, Italy; Addgene plasmid #85695) (Collavin et al., 2004), pRL-SV40 (SV40-Renilla luciferase; Promega), pTJK482 (GATA1), pTJK422 (SIX2), pTJK299 (SIX1) or WCC43 (EV). For each condition, three or four independent wells were transfected. Two days post-transfection, cells were harvested and luciferase quantitated using dual luciferase reporter assays (Promega) according to the manufacturer's instructions on a Perkin Elmer Victor X3 Multilabel Reader. Firefly luciferase was normalized to *Renilla* luciferase with entire experiment conducted in triplicate.

Proximity biotin labeling assay

Biotin ligase BirA* (TJK368) versus BirA*-SIX2 (TJK421) lentivirus was transduced into TF1 cells (MOI=2; ∼90% GFP$^+$). Cultures were

Development (2020) 147, dev177022. doi:10.1242/dev.177022

expanded for 3 days, then incubated in 50 µM biotin 24 h prior to harvesting total cell lysate in RIPA buffer containing protease inhibitors. Lysate (200 µg) was incubated with streptavidin-coated agarose beads (Sigma-Aldrich) at 4°C, washed three times prior to elution by boiling in Laemmli buffer (Sigma-Aldrich) followed by western blot analysis with anti-BirA (59938, Novus Biologicals; 1:500) or anti-GATA1 (sc-266, Santa Cruz Biotechnology; 1:200) antibodies.

Co-immunoprecipitation assay

HEK293T cells cultured in six-well dishes were transfected with a total of 2 µg DNA consisting of 1 µg pTJK482 (GATA1) with 1 µg pTJK438 (3xMYC-SIX1), pTJK477 (3xMYC-SIX2) or pWCC43 empty vector (EV) to maintain equal total DNA quantity in all transfections. Cells were lysed and protein quantitated using a Bradford assay. For immunoprecipitation, 500 µl protein lysates (1 mg/ml) were incubated overnight in the presence of 20 µl pre-washed anti-Myc magnetic beads (88842, Thermo Fisher Scientific). Samples were washed three times with co-immunoprecipitation buffer [25 µM Tris-HCl (pH 7.4), 150 µM NaCl, 1 mM EDTA, 1% NP40, 3% glycerol, protease inhibitors] and once with PBS before elution in Laemmli buffer (Sigma-Aldrich) and analysis by western blot.

Proximity ligation assay (PLA)

Wild-type or SIX1 knockout TF1 were resuspended in PBS at a concentration of 2×10^6/ml, 100 µl was centrifuged in a Cytospin3 at 5 ***g*** for 5 min. Cells were dried at room temperature prior to fixation in 4% paraformaldehyde for 10 min. Slides were then washed three times in PBS before a 10 min incubation in permeabilization buffer (50 mM NaCl, 3 mM $MgCl_2$, 10 mM HEPES, 200 mM sucrose, 0.5% Triton X-100). After washing, cells were incubated in a humidity chamber at 37°C for 1 h in PLA blocking buffer. The PLA assay (Duolink 92101, Millipore Sigma) was performed according to manufacturer's instructions using primary antibodies against SIX1 (1:50, 12891S, Cell Signaling Technology) and GATA1 (1:50, 60011-1, ProteinTech). Cells were imaged on using a Zeiss Axioplan microscope with appropriate filters for DAPI and PLA signal at 40× magnification and processed using ImageJ software.

CRISPR-engineered cell lines

To generate GATA1 and SIX1 knockout TF1 cell lines, sgRNA targeting exon 3 and exon 1, respectively, were cloned into PX459 and DNA electroporated in SE buffer using program DN-100 in the Amaxa nucleofector 4D system (Lonza). Following puromycin selection, single cell clones were generated via plating at 0.5 cells/well in 96 well plates. Knockout cell lines were confirmed by sequence and western blot. Given similar gain-of-function phenotypes and availability of a robust SIX1-specific antibody capable of detecting endogenous SIX1, we focused on SIX1 for knockout cell line generation. For inducible dCas9-KRAB parental cell line, TF1 cells were transduced with pHAGE TRE dCas9-KRAB lentivirus (Kearns et al., 2014), G418 selected and single cell clones generated. SIX1 TSS targeting sgRNA (sgRNA13), which spans the SIX1 transcriptional start site was cloned into plenti SpBsmBI sgRNA Puro vector (pTJK301) or a modified version of plenti SpBsmBI sgRNA Puro vector (pTJK466) engineered to contain U-A flip and extended hairpin reported to improve targeting (Chen et al., 2013). Corresponding lentiviruses were transduced into TF1 dCas9-KRAB cells to generate inducible SIX1 knockdown cell lines. SIX1 knockdown efficiency was assessed by western blot analysis following 3-10 days doxycycline treatment.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.G.F., T.J.K.; Methodology: M.C., R.B., C.L.E., C.S.S., M.K., N.G.F.; Validation: M.C., C.L.E., M.K.; Formal analysis: M.C., R.B., C.L.E., C.S.S., M.K., C.I.C., N.G.F., T.J.K.; Investigation: M.C., R.B., C.L.E., C.S.S., M.K., J.A.C., N.G.F.; Resources: N.G.F., T.J.K.; Writing - original draft: N.G.F., T.J.K.; Writing - review & editing: M.C., R.B., C.L.E., J.A.C., A.P., C.I.C.; Supervision: A.P., N.G.F., T.J.K.; Project administration: N.G.F., T.J.K.; Funding acquisition: N.G.F., T.J.K.

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Supplementary information

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