GLUT12 deficiency during early development results in heart failure and a diabetic phenotype in zebrafish

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Abstract
Cardiomyopathies-associated metabolic pathologies (e.g., type 2 diabetes and insulin resistance) are a leading cause of mortality. It is known that the association between these pathologies works in both directions, for which heart failure can lead to metabolic derangements such as insulin resistance. This intricate crosstalk exemplifies the importance of a fine coordination between one of the most energy-demanding organs and an equilibrated carbohydrate metabolism. In this light, to assist in the understanding of the role of insulin-regulated glucose transporters (GLUTs) and the development of cardiomyopathies, we have developed a model for \textit{glut12} deficiency in zebrafish. GLUT12 is a novel insulin-regulated GLUT expressed in the main insulin-sensitive tissues, such as cardiac muscle, skeletal muscle, and adipose tissue. In this study, we show that \textit{glut12} knockdown impacts the development of the embryonic heart resulting in abnormal valve formation. Moreover, \textit{glut12}-deficient embryos also exhibited poor glycemic control. Glucose measurements showed that these larvae were hyperglycemic and resistant to insulin administration. Transcriptome analysis demonstrated that a number of genes known to be important in cardiac development and function as well as metabolic mediators were dysregulated in these larvae. These results indicate that \textit{glut12} is an essential GLUT in the heart where the reduction in glucose uptake due to \textit{glut12} deficiency leads to heart failure presumably due to the lack of glucose as energy substrate. In addition, the diabetic phenotype displayed by these larvae after \textit{glut12} abrogation highlights the importance of this GLUT during early developmental stages.

Introduction
Cardiovascular pathologies are a leading cause of mortality (Pagidipati & Gaziano 2013). Heart failure constitutes the primary cause of death in developed countries and its occurrence increases rapidly in developing countries (Sliwa \textit{et al.} 2005, Lopez \textit{et al.} 2006). As the heart is one of the most energy demanding tissues, heart
failure is tightly correlated with alterations in cardiac metabolism. In this regard, pathologies impacting metabolism and leading to alterations in glycemic control present one of the main risk factors for developing heart failure (Haslam & James 2005). Specially, type 2 diabetes (T2D) is strongly associated with heart failure (Bell 2003). Patients with T2D present an increased incidence of heart failure ranging from two- to fivefold higher than nondiabetic patients (Kannel & McGee 1979). The main insulin-regulated glucose transporter (GLUT) expressed in peripheral tissues such as cardiac and skeletal muscle and adipose tissue is GLUT4 (Scheepers et al. 2004). In the presence of insulin, GLUT4 translocates to the plasma membrane, mediating the postprandial glucose uptake. Surprisingly, GLUT4-deficient mice did not develop hyperglycemia, and skeletal muscle tissue showed an increased glucose uptake in response to insulin (Katz et al. 1995, Stenbit et al. 1996), suggesting that there might be other insulin-sensitive GLUTs still to be identified. Interestingly, years later when it was discovered, it was found that a newly discovered GLUT named GLUT12 is expressed in the main insulin-sensitive tissues and shares motifs that are known to be important for the regulation of GLUT4 in response to insulin (Rogers et al. 2002). Moreover, Stuart et al. (2009) demonstrated that in human myoblasts insulin stimulates GLUT12 translocation to the plasma membrane via PI3-K, in the same manner as GLUT4. In this light, recent data have shown that transgenic mice overexpressing GLUT12 improved peripheral insulin sensitivity, enhancing tissue-specific insulin-stimulated glucose uptake (Purcell et al. 2011). In the same work, despite that a significant enhancement of the glucose uptake by EDL, soleus, fat and heart was observed in transgenic mice overexpressing GLUT12, the heart showed the mildest improvement of all the tissues analyzed, while these transgenic mice exhibited a twofold increase in cardiac GLUT12 protein levels, one of the highest together with the brain (Purcell et al. 2011). A possible explanation to these observations was presented recently by Waller et al. (2013) showing that in contrast to what happens in other tissues (i.e. skeletal muscle and adipose tissue) GLUT12 might be functioning mainly as a basal GLUT in the heart.

These evidences indicate that further in-depth studies on the physiological role of GLUT12 will make important contributions to better understand the connection between heart failure and pathologies impacting the glucose metabolism.

In this work, we used zebrafish (Danio rerio) model to investigate the in vivo function of glut12. Zebrafish is a genetically tractable model that offers unique advantages for in vivo studies as well as a valuable tool to study diseases impacting metabolism (Seth et al. 2013). We demonstrate that zebrafish glut12 structure and tissue distribution are highly conserved and similar to its mammalian ortholog. In addition, we show how glut12 in zebrafish is regulated by insulin and AMPK. Making use of reverse genetic approaches, we induced knockdown of glut12 in zebrafish. Our results indicate that glucose uptake mediated by glut12 is essential for cardiac development because glut12-deficient embryos exhibited normal development but showed heart failure. Moreover, knockdown of glut12 led to insulin resistance and hyperglycemia being reminiscent of diabetic phenotypes. Taken together, our results indicate that glut12 is an essential GLUT in the heart where the reduction in glucose uptake due to glut12 deficiency leads to an impaired heart development. In addition, the metabolic features of the glut12 morphants indicate that glut12 is an important insulin-regulated GLUT, making it a promising tool to assist in the development of drugs for the treatment of diabetic cardiomyopathies.

Materials and methods

Zebrafish maintenance and lines

Zebrafish were reared and handled in compliance with the local animal welfare legislation and maintained according to standard protocols (http://zfinfo.org). In this work, zebrafish lines used included WT AB/TL, Tg(myl7:egfp) (Huang et al. 2003), Tg(fli1:GFP) (Lawson & Weinstein 2002), and Tg(fabp10:dsRed;ela3l:GFP) (Farooq et al. 2008), hereafter named as 2-Color Liver Insulin Acinar Pancreas (Tg(2CLIP)). For heart extraction, insulin injections and imaging embryos were previously anesthetized in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Sigma).

Sequence alignments and molecular phylogenetic analysis

Multiple sequence alignments of the deduced amino acid sequence of the zebrafish Slc2a12 were performed using CLC Main Workbench 6.9.1 (CLC Bio A/S, Aarhus, Denmark, www.clcbio.com). Phylogenetic relationships were inferred based on the deduced amino acid sequence for zebrafish Slc2a12, using the UPGMA method in CLC Main Workbench 6.9.1. For phylogenetic analysis, 5000 iterations were used to
generate a bootstrap consensus tree. We used Genomicus genome browser (Louis et al. 2013) to perform the syntenic analysis of the genomic regions carrying glut12 loci in zebrafish compared with orthologs from other species of vertebrates.

**Morpholino design and injections**

To knockdown zebrafish glut12, two morpholino oligos (Gene Tools, LLC, Philomath, OR, USA) were designed, one of them blocking the translation of zebrafish glut12 (5'-TGCATCCATGTCTCTAAGCGTTTGT-3') and the other one targeting the splice acceptor site of exon 2 (5'-CCTTTCCCTAAATGACTAATCTGAT-3'). Morpholinos were reconstituted in RNase-free water, according to manufacturer's instructions. A standard control morpholino (5'-CCTTCAATCGTTAATATTATA-3') (Gene Tools, LLC) was injected at the same concentration as the glut12 MOs. MOs were diluted to a concentration of 0.5 mM in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES (pH 7.6)), and 1 nl was injected at the one-cell stage using a Femtojet injector (Eppendorf, Hamburg, Germany).

**RNA isolation, cDNA synthesis, RT-PCR, and qPCR**

The embryos were collected at different stages (24, 48, 72, 96, and 120 hpf), sampling 20 embryos per condition, using 1 ml of QIAzol Lysis Reagent (Qiagen) and stored at −80 °C until its RNA isolation. Also, to study the tissue distribution of slc2a12, different tissues from adult zebrafish (brain, eye, heart, intestine, liver, muscle, ovary, skin, spleen, and testis) were carefully extracted. The zebrafish were killed using an overdose of anesthesia (tricaine) and stored in RNAlater solution at 4°C. The zebrafish were killed using an overdose of anesthesia (tricaine) and stored at 4°C. The embryos were collected at different stages (24, 48, 72, 96, and 120 hpf), sampling 20 embryos per condition, using 1 ml of QIAzol Lysis Reagent (Qiagen) and stored at −80 °C until its RNA isolation.

mRNA expression levels were normalized against the expression of ppia as a housekeeping gene. The primer sequences used in this study are given in Supplementary Table 1, see section on supplementary data given at the end of this article.

**Purification of hearts from zebrafish embryos**

Extraction of hearts from 48 and 72 hpf zebrafish embryos was accomplished as described previously (Burns & MacRae 2006). A total of 150–200 hearts were used per sample. After extraction the hearts were kept in QIAzol Lysis Reagent (Qiagen) at −80 °C until its RNA isolation.

**Heartbeat rate and analysis**

To quantify the length of the cardiac cycle in zebrafish embryos and to study the differences between heartbeat rate in glut12 morphants and WT, a Hamamatsu C9300-221 high-speed CCD camera was used following the protocol described by Tessadori et al. (2012). To quantify heart rhythm, we drew kymographs for atrium or ventricle of WT and morphant embryos at 48 and 72 hpf. A total of 9–18 embryos were recorded for each condition and a total of 20 cardiac cycles were measured per embryo using ImageJ 64 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/; 1997–2014).

**Insulin and metformin administration and glucose measurements**

Insulin was injected into the zebrafish larvae as described previously (Marín-Juez et al. 2014b). For exposure to 250 μM metformin, zebrafish embryos were dechorionated at 48 hpf and treated with the compound for 48 h at 28 °C. Metformin was added to the water. Glucose measurements were performed using a fluorescence-based enzymatic detection kit (Biovision, Mountain View, CA, USA) as previously described (Jurczyk et al. 2011).

**Imaging**

Bright-field images were obtained using a Leica M165C stereomicroscope equipped with a DFC420C digital color camera (Leica Microsystems, Wetzlar, Germany). For fluorescent image acquisition, a Leica MZ16FA stereo fluorescence microscope equipped with a DFC420C digital color camera (Leica Microsystems) was used except in the case of fluorescent pictures of the valves, for which a Leica TCS SPE confocal laser scanning microscope
results

characterization of zebrafish glut12 ortholog

The zebrafish glut12 gene maps to chromosome 12 and shares a high similarity with human GLUT12 in its genomic sequence, both containing five exons (Supplementary Figure 3, see section on supplementary data given at the end of this article). Phylogenetic analysis to determine the conservation degree of the putative zebrafish Glut12 protein showed that it clustered with GLUT12 sequences from other vertebrates, being more closely related to human GLUT12 than to human GLUT10, another closely related Class 3 GLUT (Supplementary Figure 1A).

Comparison of the zebrafish Glut12-deduced amino acid sequence with those from other vertebrates showed a high percentage of sequence identity in all the cases (Supplementary Table 2, see section on supplementary data given at the end of this article and Supplementary Figure 1B). In addition, comparison of the genomic regions carrying the GLUT12 loci showed that the genes flanking zebrafish glut12 are syntenic across the different species analyzed, confirming its orthology among vertebrates (Supplementary Figure 2A).

Next, to address whether tissue expression of zebrafish glut12 is similar to that in humans using qPCR we analyzed the mRNA levels in a number of tissues extracted from adult zebrafish. This analysis revealed that, as in humans, zebrafish glut12 expression is widely distributed over various organs with the highest expression levels in skeletal and cardiac muscles (Supplementary Figure 2B). It has been shown by in situ hybridization that glut12 is expressed during the early development in the midbrain, eye, somites, and CNS (Thise & Thise 2004, Tseng et al. 2009). As we failed to reliably detect glut12 expression in the embryonic heart by in situ hybridization, we extracted the hearts of zebrafish embryos at 48 and 72 hpf. RT-PCR analysis of the RNA extracted from these samples confirmed the expression of glut12 in the zebrafish heart at these stages (Supplementary Figure 2C).

insulin and metformin stimulate glut12 expression

Human GLUT12 translocation to the plasma membrane in skeletal muscle is stimulated by insulin (Stuart et al. 2009). To better characterize the mechanisms regulating zebrafish glut12, we stimulated zebrafish larvae with insulin using a newly developed technique (Marín-Juez et al. 2014b). Zebrafish larvae at 4 days post fertilization (dpf) were injected into the caudal aorta with 100 nM and
1 µM human recombinant insulin. qPCR analysis of glut12 expression was performed in zebrafish larvae at 0.5, 1, 2, and 4 h post injection (hpi). Our results confirmed that injection of 100 nM or 1 µM insulin significantly stimulated glut12 expression at 0.5 and 4 hpi (Fig. 1A). In mammals, GLUT4 transcription is known to be stimulated via AMP-activated protein kinase (AMPK; Jorgensen et al. 2006). As glut4 is not present in zebrafish (Tseng et al. 2009, Martin-Juez et al. 2014a), we hypothesized that zebrafish glut12 may be functioning as the main insulin-regulated GLUT and therefore being regulated by similar mechanisms. To test this hypothesis, we stimulated zebrafish embryos with 250 µM metformin, an indirect AMPK activator (Zhou et al. 2001, Viollet et al. 2012). qPCR analysis confirmed that glut12 expression was significantly stimulated after metformin administration, resembling observations of mammalian GLUT4 (Fig. 1B). These evidences suggest that zebrafish glut12 expression is regulated via insulin and AMPK signaling pathways, supporting the notion that in the absence of glut4, glut12 in zebrafish could be the main insulin-regulated GLUT.

**glut12 deficiency causes impaired cardiac development**

To further study its physiological role, we resorted to reverse genetic approaches to abrogate glut12. The knockdown was performed using an antisense morpholino designed to interfere with the splicing between exons 2 and 3 (splice MO) (Supplementary Figure 3A, see section on supplementary data given at the end of this article). To ensure the specificity of this morpholino, we also designed a translation-blocking morpholino (ATG MO) (Supplementary Figure 3A). The embryos injected with the splice MO developed normally with exception of the appearance of heart edema at 72 hpf (Fig. 2A) and onwards (Supplementary Figure 3B). This was phenocopied by embryos injected with the ATG MO, confirming the specificity of the phenotype (Fig. 2A). To assess the extent of the splice-blocking activity of the splice MO, we carried out RT-PCR analysis of zebrafish embryos injected either with the control or with the splice MO from 24 to 120 hpf. Our results confirmed the efficiency of the morpholino showing the appearance of an aberrant splice product at all the different stages studied (Fig. 2B). In view of its efficiency and the possibility to easily confirm the knockdown, we chose to use the splice MO (hereafter named glut12 MO) for the rest of the study.

We then investigated whether the glut12 deficiency might be causing alterations in the heart development prior to the appearance of the cardiac edema. For this purpose, we injected the glut12 MO in Tg(myl7:eGFP) zebrafish, which allows in vivo visualization of myocardial cells. By 48 hpf, morphant embryos showed defects in chamber morphology, revealing that a large proportion of morphant embryos presented alterations in the loop formation exhibiting either abnormal (47%) or no looping

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**Figure 1**

**glut12 expression** is stimulated by insulin and metformin. (A) Zebrafish larvae were injected with human recombinant insulin at 100 nM and 1 µM in the caudal aorta at 4 dpf. Control embryos were injected with PBS. RNA samples for qPCR analysis were taken at 0.5, 1, 2, and 4 h after injection and glut12 mRNA levels were measured. (B) Relative glut12 expression after the exposure to metformin for 48 h at 250 mM. Data (mean ± s.e.m.) are derived from a combination of four biological replicates (n = 5 larvae per group) and expressed relative to the control, which is set to 1. *Significant differences between treatments (P<0.05).
In some cases reverse looping was observed, but despite that the incidence was slightly higher (5%), this phenomenon could be observed also in control embryos (2%) (Fig. 2C). The heart loop formation begins by approximately 36 hpf and is complete by 48 hpf (Bakkers 2011). This fact, together with the previous observations, led us to hypothesize that glut12 deficiency would cause a reduction in glucose uptake by the heart, resulting in a reduced growth and proliferation of cardiac cells impairing the development of the heart. In this regard, to confirm that the observed phenotype was not restored after 48 hpf, we injected Tg(myl7:eGFP) embryos with the glut12 MO. Phenotypes were classified according to the looping. (D) Further characterization of these embryos was carried out and the morphology of these hearts was studied at 72 hpf. 35 hearts of each type were analyzed. Scale bar indicates 150 μm. (E) Tg(fli1:GFP) embryos were injected with control and glut12 MO and vasculature of the tail region was visualized at 48 and 72 hpf. (F) Tg(2CLIP) embryos were injected with the control and glut12 MO. Liver (yellow-orange) and pancreas (green) were visualized at 96 hpf. A full colour version of this figure is available via http://dx.doi.org/10.1530/JOE-14-0539.

Overall, our results confirm that glut12 deficiency alters heart development leading to the formation of an underdeveloped cardiac structure.

### Loss of glut12 leads to alterations in atrial and ventricular contraction resulting in arrhythmias

In view of the previous observations, we hypothesize that an underdeveloped heart may exhibit alterations in the heart rate and chamber contraction. To characterize these parameters, we analyzed the heart rhythm of glut12 morphants by high-speed video imaging, quantifying heart rate and fractional sorting as described by Tessadori et al. (2012). As we observed that atrial and ventricle contractions were coupled in both control and morphant

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**Figure 2**

glut12 knockdown leads to impaired heart development. (A) Zebrafish embryos at 72 hpf injected with either ATG or control MOs exhibited heart edema (black arrowhead). White dotted boxes indicate the regions shown in the inserts. (B) RT-PCR analysis of larvae from 24 to 120 hpf injected with control and glut12 MO to assess the extent of the splice-blocking activity of the splice MO. (C) The penetrance of the glut12 knockdown was studied observing the different cardiac phenotypes present in the Tg(myl7:eGFP) embryos injected with the glut12 MO. Phenotypes were classified according to the looping. (D) Further characterization of these embryos was carried out and the morphology of these hearts was studied at 72 hpf. 35 hearts of each type were analyzed. Scale bar indicates 150 μm. (E) Tg(fli1:GFP) embryos were injected with control and glut12 MO and vasculature of the tail region was visualized at 48 and 72 hpf. (F) Tg(2CLIP) embryos were injected with the control and glut12 MO. Liver (yellow-orange) and pancreas (green) were visualized at 96 hpf. A full colour version of this figure is available via http://dx.doi.org/10.1530/JOE-14-0539.
embryos (data not shown), we performed all the high-speed imaging-based measurements in the atrium of zebrafish embryos at 48 and 72 hpf. Heart rhythm was quantified by kymographs obtained from the high-speed videos. These kymographs showed that the time required for a full cardiac cycle in \(\text{glut12}\) morphants was significantly extended (Fig. 3A). Next, based on these kymographs we quantified the cardiac cycle length and observed that the heart rate was significantly reduced (bradycardia) in \(\text{glut12}\) morphants at 48 and 72 hpf (Fig. 3B and C). In addition, cardiac length analysis confirmed the bradycardia and revealed irregular heart rhythm in \(\text{glut12}\) morphants that could be observed in some cases at 48 hpf (Fig. 3D) and clearly at 72 hpf (Fig. 3E). These observations indicate that the delayed cardiac development caused by the deficiency in \(\text{glut12}\) leads to heart failure.

**glut12 morphants exhibit blood toggling between heart chambers as a consequence of abnormal valve formation**

Low heart rates have been described to lead to abnormal valve formation (Vermot et al. 2009). To investigate this effect, we analyzed high-speed imaging videos of control and \(\text{glut12}\) morphant embryos at 72 hpf, because at this stage valves should be fully formed preventing any retrograde flow fraction (RTT; Vermot et al. 2009). Interestingly, we observed that while in control embryos blood flow was unidirectional following atrial and ventricular contractions, morphant embryos exhibited blood toggling between the atrial systole and the ventricular diastole (Fig. 4A, Supplementary Movies 1 and 2, see section on supplementary data given at the end of this article). To confirm whether the alterations in heart rate affected valve morphogenesis in \(\text{glut12}\) morphants causing blood toggling, we used the \(\text{Tg}(\text{fli1:GFP})\) line to visualize the valve formation at 72 hpf. While in control embryos we could observe the valves in between both cardiac chambers, these were absent in \(\text{glut12}\) morphants (Fig. 4B). Vermot et al. (2009) demonstrated that alterations in the heart rate cause valve dysgenesis via down-regulation of \(\text{klf2a}\). To confirm this, we carried out qPCR analysis in hearts extracted from control and \(\text{glut12}\) morphant embryos at 72 hpf. Our results confirmed that \(\text{klf2a}\) expression was significantly down-regulated in morphant embryos (Fig. 4C). Taken together, these data indicate that the alterations in the heart rate as a consequence of the \(\text{glut12}\) deficiency down-regulate \(\text{klf2a}\) leading to abnormal valve formation.

**Abrogation of \(\text{glut12}\) leads to hyperglycemia and insulin resistance**

We set out to further investigate the physiological consequences of \(\text{glut12}\) abrogation. In view of the apparent
lack of glut4 in zebrafish and the previous observation indicating that insulin stimulates glut12 expression, we hypothesize that a deficiency in glut12 may lead to alterations in glycemic control of the larvae. To test this hypothesis, we measured free glucose levels in control and glut12 MO injected embryos from 2 to 5 dpf.

In agreement with previous observations, free glucose levels appeared to diminish after 2 dpf, increasing again from 3 to 5 dpf (Jurczyk et al. 2011, Gut et al. 2013). This could correspond to an initial increase in glucose levels that might drive the maturation of beta pancreatic cells as observed in mammals (Hellerstrom & Swenne 1991, A)

Figure 4

glut12 morphants exhibit backflows of blood as a consequence of abnormal valve formation. (A) Bright field images recorded by high-speed imaging comparing control and glut12 morphant embryos showing a schematic representation of a cardiac cycle in zebrafish embryos. A zebrafish embryonic heart at 72 hpf is highlighted in white dotted contour and valves are highlighted in white dashed contour. Red ellipses, erythrocytes following normal flow; yellow arrows, normal blood flow; blue ellipses, erythrocytes fraction returning to the atrium; white arrows, blood backflow (For more detailed information see Supplementary Movies 1 and 2). (B) To better visualize the valve morphology in morphant embryos compared to the control we used Tg(fli1:GFP). White boxes indicate the regions magnified in the sections i and ii. Scale bar indicates 50 μm. (i) High magnifications of the AVC regions. # indicates the closure of the AVC canal by the valves. The white arrow indicates the abnormal valve morphology and the consequent opening of the AVC. (ii) Schematic representation of the AVC regions. (C) Expression of klf2a was analyzed by qPCR. RNA was extracted from control and glut12 morphants at 72 hpf. Data (mean ± S.E.M.) are combined from 4 biological replicates (n = 150–200 hearts per group) and expressed relative to control, which is set to 1. *P<0.05. V, ventricle; A, atrium; AVC, atrio-ventricular canal; OFT, outflow tract. A full colour version of this figure is available via http://dx.doi.org/10.1530/JOE-14-0539.
Cao et al. 2004), dropping at 3 dpf and increasing afterwards, coinciding with the functional onset of the hepatic gluconeogenesis (Gut et al. 2013). Our results showed that between 3 and 5 dpf, glut12-deficient embryos presented significantly higher free glucose levels (Fig. 5A). Next, to investigate whether the knockdown of glut12 would be translated into a reduced insulin sensitivity, we measured free glucose levels in 4 dpf larvae at 15, 30, 60, and 120 min post injection of 1 nl of 100 nM human insulin. Interestingly, in glut12-morphant larvae, insulin administration caused no significant reduction in free glucose levels, in contrast to control embryos where the expected hypoglycemic effect was observed (Fig. 5B and Supplementary Figure 4, see section on supplementary data given at the end of this article).

Altogether, these data suggest that glut12 abrogation causes a reduction in glut12-mediated glucose uptake causing hyperglycemia and insulin resistance, resembling a diabetic phenotype.

Transcriptomic profile confirms the observed cardiac and diabetic phenotype and reveals tissue-specific effects upon glut12 knockdown

To better understand the impact of glut12 deficiency at the whole embryo level, we carried out an RNAseq-based transcriptome analysis of 4 dpf glut12-morphant larvae compared with control larvae. To analyze the data, we set significance cut-offs at 1.5-fold change at \( P<0.05 \) (FDR adjusted \( P \) value). In this way, we obtained 1099 up-regulated genes and 290 down-regulated genes. Next, to further characterize the differentially expressed genes (DEGs), we performed a GO analysis for functional classification (Supplementary Tables 3, 4, and 5, see section on supplementary data given at the end of this article). In addition, RNA-seq data were validated by qPCR, observing the same effects on the expression of the genes analyzed by both techniques (Supplementary Figure 5, see section on supplementary data given at the end of this article).

The study of GO terms showed that glut12 deficiency altered biological processes involved in the development of the main organs responsible for glycemic control, which are commonly affected in diabetes (e.g., heart, liver, kidney, and skeletal muscle). In addition, other categories involving important pathways related to insulin signaling and action appeared dysregulated, such as the JAK–STAT and protein kinase cascades. When looking at cellular component and molecular function GO terms, we found an enrichment in categories involved in neural development. For more detailed analysis of the transcriptomic profile of the glut12 morphants, we selected genes important for cardiac, hepatic, and neural function and development and insulin signaling pathway, as well as genes involved in metabolism and diabetes and/or insulin resistance (Fig. 6). Among DEGs involved in the insulin signaling pathway, a number of genes important to this pathway appeared up-regulated (insr, irs2, pik3ca, pdk2). Moreover, we observed a number of DEGs known to be important for the function and development of the heart (klf2a, actc1a, gata4, srv, srx10a, tcp, dbh, tek), liver (mtf1, stat3, rgs3, ppar6) and brain (map1a, gamt, nos1, garh2, tph1a, gpr3, ddita). Notably, we found that several genes known to be dysregulated in
diabetes or in insulin resistance (nod1, pgc1, il6st) were up-regulated in glut12 morphants. Furthermore, genes involved in lipid metabolism (fabp1a, fabp7) and gluconeogenesis (fbp2, pck1) were down-regulated in glut12-deficient larvae. Finally, we found two GLUTs down-regulated glut5 and glut12, the latter confirming the efficiency of the splice-blocking morpholino.

**Discussion**

Despite the fact that the heart can use a broad range of substrates as fuel, its metabolic machinery is finely regulated to ensure an optimal usage of the energy provided by these substrates. Aberrant metabolism is a common result of heart failure, for which cardiomyocytes resort to the usage of different substrates to fulfill the energetic needs of this metabolically demanding organ (van der Vusse et al. 2000, van Bilsen et al. 2004). Therefore, pathologies altering the control of metabolism (e.g., diabetes and insulin resistance) have critical consequences contributing to the development and worsening of cardiomyopathies (Bell 2003b). One of the most recently discovered GLUTs, GLUT12, has been shown to be regulated by insulin in skeletal muscle (Stuart et al. 2009) and importantly has been described as a basal cardiac GLUT (Waller et al. 2013). Therefore, to gain insights into the physiological role of this GLUT, in this work we set out to study the functional consequences of glut12 abrogation. As a result we could show how glut12 abrogation impairs cardiac development, altering the contraction rate that in turns leads to alterations in the valvulogenesis. Moreover, we observed that glut12-deficient larvae were insulin resistant, exhibiting reduced insulin sensitivity and hyperglycemia, features characteristic of the diabetic phenotypes.

Both GLUT4 and GLUT12 have been shown to perform an important role in the cardiac muscle. Studies using a canine model for chronic heart failure showed that the expression of both GLUT4 and GLUT12 is affected in specific myocardial structures (Ware et al. 2011).
Moreover, Glut4 deficiency in the heart has been reported to cause cardiomyopathies (Domenighetti et al. 2010). Supporting this notion, our data clearly show that the knockdown of glut12 impairs embryonic heart development leading to alterations in contractility and bradycardia. Embryonic heart development is a tightly regulated process for which the energy source has been found to be a key determinant. During early development, cardiomyocyte precursors rely mainly on glycolysis to obtain energy (Chung et al. 2007, 2010). In addition, it has been described that proper heart formation is the result of a combination of genetic and mechanical factors that result in the proper development of the cardiac structures (Hove et al. 2003, Vermot et al. 2009). Valvulogenesis appears to be especially sensitive to alterations in this mechanogenetic equilibrium. Recently, Vermot et al. (2009) demonstrated that alterations in the RTT result in defective valve formation. Moreover, in the same study it was shown that variations in the heart rate lead to variations in the RTT via down-regulation of klf2a, causing valve dysgenesis as a consequence (Vermot et al. 2009). KLF2’s responsiveness to the blood flow is well conserved and it has been found expressed in regions subjected to high shear forces in human, chicken, and zebrafish hearts (e.g., the site of valve formation) (Dekker et al. 2002, Groenendijk et al. 2004, Vermot et al. 2009). As in mammals, glut12 is found highly expressed in the heart of zebrafish and its abrogation resulted in clear alterations of the heart development probably as a consequence of the energy imbalance experienced by the cardiac muscle due to deficient glucose uptake. In view of this, we propose that the deficiency in glucose uptake caused by glut12 abrogation impaired the embryonic heart development altering the loop formation, the contractility of myocardial cells, and the heart rate. This, in turn, altered the shear forces in the cardiac endothelium causing klf2a down-regulation and as a consequence valve dysgenesis (Fig. 7). Moreover, our deep sequencing data confirmed the down-regulation of klf2a and showed that the expression of a number of genes important for cardiac functioning and development was affected. Interestingly, gata4 appeared up-regulated in our data set. Gata4 overexpression has been reported to cause cardiac hypertrophy (Liang et al. 2001). During physiological cardiac

Figure 7
Proposed model for cardiac glut12 deficiency. Cardiac glucose uptake is reduced as a consequence of the glut12 abrogation. This impact on energy balance impairs the cardiac development altering the contractility of the myocardial cells and the heart rate. Embryonic hearts exhibiting poor looping and bradycardia have a reduced RTT down-regulating klf2a, which in turn causes valve dysgenesis. A full colour version of this figure is available via http://dx.doi.org/10.1530/JOE-14-0539.
hypertrophy, cardiac metabolism switches from glycolysis to fatty acid β-oxidation as the main source of energy (Lorell & Grossman 1987). However, in adult hearts experiencing pathophysiological hypertrophy, cardiac metabolism reverts to glycolytic metabolism resembling the fetal scenario (Lorell & Grossman 1987, Depre et al. 1998, Razeghi et al. 2001). In our case, gata4 up-regulation in glut12 morphants could reflect an attempt to switch to a less glucose-dependent cardiac metabolism. In our opinion, a scenario where gata4 up-regulation may be reflecting pathological hypertrophy is most likely. The observed down-regulation in glut12 morphants of snx10a, dbh, and tek supports this idea, because lack or deficiency of these genes has been reported to alter cardiac function, in some cases leading to heart failure (Swoap et al. 2004, Tachibana et al. 2005, Chen et al. 2012). We performed deep sequencing analysis at whole-embryo level to assess the global impact of glut12 deficiency. Future transcriptomic studies focused on the heart in a tissue-specific manner will be needed to further study the observed phenotype.

GLUT12 has been proposed to be a secondary GLUT that complements GLUT4 mediating, insulin-stimulated glucose uptake (Stuart et al. 2009, Purcell et al. 2011). It has been shown that, in skeletal muscle, GLUT12 translocates to the plasma membrane together with GLUT4 after insulin stimulation (Stuart et al. 2009). These observations could also explain why Glut4-knockout mice still showed insulin-stimulated glucose uptake and did not develop hyperglycemia (Katz et al. 1995, Stenbit et al. 1996). Moreover, Stenbit et al. showed later that Glut4 heterozygote knockout mice displayed reduced insulin sensitivity in the skeletal muscle, hyperinsulinemia, and hyperglycemia (Stenbit et al. 1997). In this work, the authors propose that, while heterozygous mice have Glut4 until the age of 8 weeks and then experience a decrease in Glut4 content, Glut4-null mice have complete lack of the transporter developing and activating other mechanisms to compensate for this deficiency (Stenbit et al. 1997). One of these mechanisms could be Glut12.

Current data support the notion that in zebrafish glut4 has been lost (Tseng et al. 2009, Marín-Juez et al. 2014a), making it an attractive model for the study of glut12, which could be functioning as the main insulin-regulated GLUT. Zebrafish glut12 presents a high degree of conservation compared with its human counterpart in its protein structure, syntenic distribution, and tissue expression pattern. Moreover, our data also show that, in agreement with the observations in mammals, its expression is regulated by insulin. Recently, it has been published by our group that human insulin is effective in zebrafish larvae (Marín-Juez et al. 2014b). In that work, we showed how 4 dpf larvae injected with 100 nM insulin experienced transient hypoglycemia between 15 and 45 min post injection, becoming insulin resistant at 4 hpi (Marín-Juez et al. 2014b). Interestingly, the expression of glut12 is stimulated at 0.5 and 4 hpi, times at which zebrafish larvae are insulin sensitive and resistant respectively. The fast response observed at 0.5 hpi could indicate that glut12 expression is rapidly stimulated in the presence of insulin to ensure an efficient glucose uptake. The latter stimulation could be explained as an attempt to compensate for the hyperglycemia that these larvae experience as a consequence of the hyperinsulinemia-induced insulin resistance that they experience at 4 hpi (Marín-Juez et al. 2014b).

As in mammals GLUT4 is known to be regulated by AMPK, we hypothesized that this could be the case for glut12, assuming that in zebrafish it is functioning as the main insulin-regulated GLUT in the absence of glut4. Our data showed that stimulation with the antidiabetic drug metformin, an indirect AMPK activator (Viollet et al. 2012), stimulates glut12 expression, suggesting that like mammalian GLUT4, zebrafish glut12 could be regulated via the AMPK signaling pathway. It is worth mentioning that because one of the main targets of metformin is the mitochondrial respiratory chain, it has been proposed that the activation of AMPK is a consequence of the energy imbalance that the cells experience when exposed to the drug (Viollet et al. 2012). In view of this, we cannot rule out the possibility that the observed effects might be due to AMPK-independent mechanisms. Future in-depth studies will help to elucidate this.

Interestingly, we show that glut12 deficiency caused hyperglycemia and insulin resistance. These results are in agreement with recent data showing that mice overexpressing Glut12 presented an improved insulin sensitivity (Purcell et al. 2011) and further support that in zebrafish glut12 is an insulin regulated GLUT. In addition, the observed hyperglycemia might be a consequence of the reduced glucose uptake by tissues expressing glut12, especially the skeletal muscle since this represents a high portion of the larvae body mass.

The transcriptomic profiling showed that nod1, il6st, srf and pgc1 were up-regulated in glut12 morphants supporting the observed phenotype since activation in the case of Nod1 or increased levels in the case of Il6st have been linked to insulin resistant states (Zuliani et al. 2010, Schertzer et al. 2011). Overexpression of srf is increased in
T2D but also has been reported to cause cardiomyopathy (Zhang et al. 2001) and Pgc1α dysregulation has been extensively studied and linked to T2D. However, in the case of Pgc1α, the observations differ in a tissue specific manner, observing Pgc1α up-regulation in the liver of mouse models for T1D and T2D (Puigserver & Spiegelman 2003), and down-regulation in muscle from humans with T2D (Mootha et al. 2003, Patti et al. 2003). Moreover, Pgc1α also plays a key role as a mediator of cardiac metabolism (Lehman et al. 2000, Arany et al. 2005). As noted before, future tissue specific studies will be helpful to elucidate the specific role of pgc1α in a situation of glut12 deficiency.

It is worth mentioning that our transcriptomic analysis also showed enrichment in DEGs involved in neural function and development. Interestingly, Pujol-Gimenez et al. (2014) found that GLUT12 expression was increased in the frontal cortex of patients with Alzheimer disease, suggesting for the first time that GLUT12 might play a role in the development of Alzheimer disease.

In summary, in the present manuscript we demonstrate that glut12 deficiency causes heart failure and diabetic phenotypes during embryonic development. In addition, it has been previously hypothesized that during early embryonic development, Glut12 might be the main GLUT responsible for insulin-regulated glucose uptake (Macheda et al. 2002) since Glut4 is not expressed during early development, being only detectable at very low levels at late fetal/early postnatal life in mouse (Santalucia et al. 1992). In this regard, the zebrafish model for glut12 deficiency that we used in this study will be a unique tool to not only study fetal diabetic cardiomyopathies but also contribute to the development and discovery of new anti-diabetic drugs thanks to its characteristics as a vertebrate model with high-throughput screening possibilities.

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Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0539.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
V J-A designed and performed experiments, analyzed the data, and wrote the paper. S J-R designed and performed experiments. J B participated in the design of the study, performed the statistical analysis, and made available facilities. H P S supervised the work, wrote the paper, and made available facilities and resources. R M-J conceived the study, designed and performed experiments, supervised the work, and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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References
Burns CG & MacRae CA 2006 Purification of hearts from zebrafish embryos. BioTechniques 40 274, 276, 278 passim. (doi:10.2144/000112135)


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